Standard Procedures for Aquatic Animal Health Inspections

Developed and Edited by:

United States Fish and Wildlife Service
And
American Fisheries Society – Fish Health Section

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Chapter 1

Introduction

1.1 Introduction

A. Purpose

The purpose of this document is to serve as a set of minimum standard protocols to be followed when carrying out health inspections on aquatic animals. These inspections may be used for intrastate, interstate, or international movement of animals. Using a standard set of procedures and protocols allows fisheries managers to better compare data and make better management decisions. However, the final decision to require the use of these protocols remains in the hands of those regulating bodies requesting/requiring health inspections. This handbook is in no way is meant to provide, dictate, or supplant aquatic animal health policies of any entity, and is solely intended to be a procedural handbook.

B. Composition

This handbook reflects the combined efforts and expertise of the United States Fish and Wildlife Service Fish Health Centers and the American Fisheries Society Fish Health Section. It has been assembled by a vast array of individuals with academic and field expertise. It is a compilation of methodologies determined to be most appropriate for detecting the presence of specific pathogens during an aquatic animal health inspection. The methodologies have been taken from numerous sources, including the United States Fish and Wildlife Service's National Wild Fish Health Survey Manual, the 4th edition of the American Fisheries Society Blue Book, the 3rd edition of the Office International des Epizooties (OIE) Diagnostic Manual for Aquatic Animal Diseases, Alaska Department of Fish and Game, Fish Pathology Section Laboratory Manual, and the peer reviewed literature. Without the substantial contributions of these documents, and the individuals that assembled and bench tested them, the development of this handbook may not have been possible.

C. Design

To be effective and to meet the needs of what is a rapidly changing and expanding field, this handbook must be extremely dynamic. Every section, including this first chapter, is open to revision. The design of this handbook will allow it to grow and change as time progresses and standardized inspection techniques are required for additional pathogens or better testing methods are made available. The guidelines for introducing changes are outlined in Appendix 1. Appendix 3 summarizes the decision making process for the selected assays and pathogens so that future changes can be made efficiently.

D. Selection of Pathogens

The pathogens considered in this handbook are those that have the potential to produce severe epizootics of clinical disease, but are also known to exist in a carrier state. They are pathogens of regulatory concern, for which there are both screening and confirmatory tests available. The methodologies described herein are effective for the detection and identification of each pathogen in the absence of clinical signs.

E. Selection Of Methodologies

The appropriateness of methodologies was determined based not only on performance characteristics (sensitivity, specificity, repeatability, and reproducibility) but also on the appropriateness in a given species or population, scientific acceptance and citable reference materials, cost, availability of reagents, availability of technology, time necessary to process samples and time required before a report can be issued, sample type and its viability, manpower requirements, number of samples to be done, the existence of reference standards, and safety.

The assays specified in this handbook are of two types. Generally, the screening method is one with a proven track record for isolation of a fish pathogen or, less commonly, for direct observation of a causative agent or detection of a component of a fish pathogen. In many cases (e.g. cell culture isolation of fish viruses), these were the first methods developed for this purpose. Although many of these assays have been used for sufficient periods of time to engender confidence in their utility, few if any of the screening methods have been subjected to the formal validation process outlined by the OIE (see next section). Typically, this is because for many years, few competing assays were available for the purpose; however, there remains a need for the validation process to be applied to these screening tests. Nevertheless, specifying a single screening method ensures that the sensitivity of the assay used to detect a pathogen, while not know with precision, is relatively uniform wherever the assay is applied.

The second type of assay is a confirmatory, test used to verify the identity of a suspect agent. These assays are also in need of formal validation, but are used here only to accurately identify an agent isolated in culture or identified visually, with less regard as to their absolute level of sensitivity. Various assays may be appropriate as confirmatory tests if the specificity of each assay is high. Knowledge of the sensitivity of a confirmatory test becomes of increased importance when the assay is used to confirm a screening test that does not propagate the pathogen in culture. An example is the use of a DNA-based test (e.g. PCR) to confirm a serological test (e.g. FAT). In this case, both assays detect proxies for the actual agent. Better information on the sensitivity, specificity, repeatability and reproducibility of these assays would allow fish health workers to choose optimal combinations of such proxy assays.

F. Validity of Chosen Methodologies

Validation is the evaluation of a process to determine its fitness for a particular use. For diagnostic assays, this generally involves measuring the ability of a certain test to accurately predict the infection status of an animal from which a sample was obtained. Naturally, many factors affect the ability of any assay to achieve absolute predictive power. In recent years, the development of assays based upon molecular approaches and the increased attention paid to issues of quality assurance have led to concerns about the relative performance of various diagnostic assays for fish diseases.

The principles involved in validation of diagnostic assays for infectious diseases are included as a chapter in the Diagnostic Manual for Aquatic Animal Diseases published by the Office

International des Epizooties (OIE) in Paris, France. This material is available on the web (www.oie.int) and in hard copy from the OIE. In addition to presenting the principles involved, the OIE lists five stages for diagnostic assay validation and discusses these in some detail with references for further reading. The chapter also describes the procedures by which the important parameters of sensitivity, specificity, repeatability and reproducibility are determined within a known degree of statistical precision.

Finally, as new diagnostic tests for fish diseases become available, there is interest in comparing the relative performance of various assays. Formal validation methods provide a basis to accurately quantify the actual performance of assays used in fish health and provide information that can help to determine when newer assays should replace the established assays as screening or confirmatory methods. Validation methods can also resolve uncertainties as to the relative performance of materials from different commercial sources or molecular assays that are similar in type, but target different proteins or genes of a pathogen or that use different protocols or reagents. Unfortunately few if any fish disease detection assays have ever been validated in this way. While validation testing involves much work, considerable expense, and the assembly of sets of standard samples of known infection status, the fish health community is very much in need of this information.

G. Other Important Considerations

It should be noted that as with any inspection manual or handbook the techniques employed provide only a snapshot in time. There is no guarantee that any inspected animal or population of animals is disease free. The only statement that can be made is that at the time the animals were sampled the disease organism was not detected by the testing methods utilized. Additionally this handbook cannot cover every scenario that may occur. For this reason it remains the obligation and responsibility of the individual inspector to determine what the best sampling protocols are for a given inspection and that any inspection methods or protocols used meet the requirements of the requesting entity.

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U.S. Fish and Wildlife Service and/or the United States government. Any comparable instrument, laboratory supply or reagent may be substituted in this document if operation and performance are deemed comparable to the items specified.

Chapter 2

Sampling

2.1 Introduction

This section describes procedures for the collection of samples for both lot and facility inspections. These guidelines represent the **minimum acceptable standard** and may be superceded by applicable State, Tribal, interjurisdictional and foreign aquatic animal health protection guidelines, policies, and /or regulations.

Size and age group classifications, lot definitions, sample size, general sampling considerations, and a recommended protocol for sample collection are also included. Screening and confirmatory tests to be run on the samples collected are provided, in detail, for each pathogen in subsequent chapters of this handbook (see Contents).

The following necropsy and sample collection protocol is for four bacteria (*Aeromonas salmonicida, Yersinia ruckeri, Edwardsiella ictaluri*, and *Renibacterium salmonarium*), eight viruses (Infectious Hematopoietic Necrosis Virus, Infectious Pancreatic Necrosis Virus, Infectious Salmon Anemia Virus, Largemouth Bass Virus, *Oncorhynchus masou* Virus, Spring Viremia of Carp Virus, Viral Hemorrhagic Septicemia Virus, and White Sturgeon Herpesvirus), and four parasites (*Myxobolus cerebralis, Ceratomyxa shasta, Tetracapsula bryosalmonae,* and *Bothriocephalus acheilognathi*). A brief description of clinical signs of the diseases caused by each of these pathogens is included in this Handbook. However, references such as the AFS/FHS Blue Book (Thoesen, 1994) and others listed herein should be consulted for more detailed descriptions and information.

It is the responsibility of the inspector to evaluate the facility and select the appropriate fish from which to collect samples, check the policies and regulations of the jurisdictions requiring the inspection, and coordinate sample submission requirements with the laboratory that will be performing the assays. This will ensure recognition of the inspection results by the competent authorities. A guideline for inspection preparation is provided in 2.2.A.

The inspection criteria outlined in this chapter is based on the testing of a statistically valid number of individuals to assess the health of the entire population and is intended only to provide information for risk management involving the movement of fish from one location to another. The lack of detection of a particular pathogen resulting from an inspection indicates only that the pathogen was not isolated by the methods used on the individual fish examined on the date of sampling. It does not indicate that every fish in that population is free of that pathogen or will be free of that pathogen at any time in the future.

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2.2 Sampling

A. Guidelines for Preparation of a Fish Health Inspection

1. It is the responsibility of the inspector to obtain the appropriate information from the facility manager, receiving jurisdiction, and testing laboratory in order to assess all appropriate samples to collect from each lot at the facility. The following is a checklist of information to consider when planning and preparing for an inspection.

   Facility Information
   - Facility Water Source(s) – do any of the sources contain live fish?
   - Facility Water Temperature Regime
   - Identification of all lots of fish present at the facility at the time of inspection and during the previous 12 months (See 2.2.C for definition of Lot)
   - Origin and History of each Lot: Strain information, transfer information and previous inspection history.
   - Location of fish in each lot at the facility (by both water source and rearing unit)
   - General health history, including any therapies administered to fish during the previous 12 months.

   Receiving Jurisdiction Information
   - Name(s) of state contacts
   - State Regulations that apply
   - Regional Policy that may apply
   - Pathogens listed by regulation or policy
   - Sampling and Laboratory analysis methods required by regulation or policy
   - Does testing laboratory meet all requirements of receiving jurisdiction(s)?

2. Listed in Table 2.1 are the target fish species, size/age group and tissue to be selected for inspection for each pathogen. To the extent that inspection requirements allow for it, sampling efforts will be directed at the most susceptible species, age, temperature, and rearing units for that pathogen. For some species, strain susceptibility or resistance, as well as other performance factors can be obtained and reviewed from the National Fish Strain Registry (http://159.189.37.201/).

3. All samples shall be processed as soon as possible after collection. If the collected animals are not maintained alive before processing, samples shall be stored chilled (0-4°C) but not frozen and shall be processed as soon as possible after collection. All samples for virology must be inoculated onto cell cultures within 72 hours post-collection. Fish selected should be representative of the lot being inspected and shall include fish with lesions and moribund fish when present.

Table 2.1 - Target fish species, size/age group and tissue to be selected for inspection for each pathogen.

Organism	Common name of disease	Known Susceptible Species	Tissue for Sampling	Primary (Screening Technique)	Confirmatory Technique	Comments
Bacterial Pathogens						
Aeromonas salmonicida	Furunculosis	Any freshwater fish	Kidney	Bacterial culture of kidney on TSA or BHIA media	Fluorescent Antibody Test (FAT)	May be isolated from many species of fish, birds, and protozoan parasites
Yersinia ruckeri	Enteric Red Mouth (ERM)	Any freshwater fish	Kidney	Bacterial culture of kidney on TSA or BHIA media	FAT	May be isolated from many species of fish and birds
Edwardsiella ictaluri	Enteric septicemia of catfish (ESC)	Ictalurids	Kidney	Bacterial culture of kidney on TSA or BHIA media	FAT	
Renibacterium salmoninarum	Bacterial Kidney Disease (BKD)	Salmonids	Kidney, ovarian fluid	Direct fluorescent antibody test on kidney smear or ovarian fluids	Bacterial culture using SKDM-2 media for a total of 6 weeks *or* nested Polymerase Chain Reaction (PCR) technique	
Viral Pathogens						
Infectious Hematopoietic Necrosis Virus	IHN	Salmonids	Whole fry, viscera or kidney/spleen - depending on size, ovarian fluid	Cell culture on EPC cells for 14 days at 15° C. Followed by a 14 day blind pass.	Serum neutralization or nested PCR	Target tissues should be kidney/spleen from larger fish and ovarian fluid from spawning broodstock.
Infectious Pancreatic Necrosis Virus	IPN	Wide variety of freshwater and saltwater fish and shellfish	Whole fry, viscera or kidney/spleen - depending on size, ovarian fluid	Cell culture on CHSE-214 cells for 14 days at 15° C. Followed by a 14 day blind pass.	Serum neutralization	Target tissues should be kidney/spleen from larger fish and ovarian fluid from spawning broodstock. May be isolated from many species of aquatic organisms
Infectious Salmon Anemia Virus	ISA	Salmonids and Atlantic herring	Whole fry, viscera or kidney/spleen - depending on size; ovarian fluids	Cell culture on SHK-1 cells for 14 days at 15° C. Followed by a 14 day blind pass.	PCR technique	In addition to sampling kidney spleen, when available sample ovarian fluid from spawning broodstock. Most mortality occurs in saltwater with fluctuating temperatures
Oncorhynchus masou Virus	OMV	Salmonids	Viscera, ovarian fluids	Cell culture on CHSE-214 cells for 14 days at 15° C. Followed by a 14 day blind pass.	Send to reference lab for confirmation	Target tissues should be kidney/spleen from larger fish and ovarian fluid from spawning broodstock. Only known to occur in Japan
Viral Hemorrhagic Septicemia Virus	VHS	Salmonids, pike, turbot, herring, pilchard	Kidney/spleen	Cell culture on EPC cells for 14 days at 15° C. Followed by a 14 day blind pass.	PCR	In addition to sampling kidney spleen, when available sample ovarian fluid from spawning broodstock.

Organism	Common name of disease	Known Susceptible Species	Tissue for Sampling	Primary (Screening Technique)	Confirmatory Technique	Comments
White Sturgeon Herpesvirus	WSHV	White sturgeon, possibly shortnose sturgeon	Kidney/spleen, ovarian fluids	Cell culture on WSS-2 cells for 14 days at 20° C. Followed by a 14 day blind pass.	Send to reference lab for confirmation	
Largemouth Bass Virus	LMBV	Centrarchids and ecocids	Kidney/spleen/swim bladder	Cell culture on FHM or BF-2 cells for 14 days at 20-25° C. Followed by a 14 day blind pass.	PCR	
Spring Viremia of Carp Virus	Infectious carp dropsy	Cyprinids, also brown trout, pike, shrimp and copepods	Kidney/spleen	Cell culture on EPC cells for 14 days at 20-25° C. Followed by a 14 day blind pass.	Serum neutralization	Most easily isolated in the spring during and for several weeks after epizootics.
Parasite Pathogens						
Myxobolus cerebralis	Whirling Disease	Salmonids	Cranial cartilage (entire head or wedge/core sample from larger fish	Pepsin-trypsin digest	Histological observation of spores/lesions consistent with infection in cranial cartilage or nested PCR	For a facility inspection only one lot of the most susceptible species on each water source need be inspected. When possible select fish that have been on that water supply, while at a susceptible age, for a minimum of 1800 degree-days or for six (6) months.
Ceratomyxa shasta	Ceratomyxosis	Salmonids	Intestine (posterior)	Wet mounts of intestinal scraping	Detection of spores or PCR	When possible select fish 1) in earth ponds or ponds receiving untreated surface water, 2) that have been on that water supply for a minimum of six (6) months and 3) that are moribund or lethargic.
Tetracapsula bryosalmonae	Proliferative Kidney Disease (PKD)	Salmonids	Kidney	Smears of kidney stained with Leishman-Giemsa or Lectin	Histology	When possible: 1) select fish from earth ponds or raceways receiving untreated surface water, 2) sample moribund fish and 3) conduct sampling during summer or early fall months.
Bothriocephalus acheilognathi	Asian Tapeworm	Cyprinids, silurids, poeciliids, percids, centrarchids, gobiids, cyprinodontids	Intestine (anterior one third)	Visualization of cestode with pyramidal scolex in the semi-contracted state	Positive identification by use of a key	Late summer and fall sampling optimal for detection.

Table 2.1 (Continued) - Target fish species, size/age group and tissue to be selected for inspection for each pathogen.

4. Samples are collected by, submitted by, and accepted from a federally accredited veterinarian, a state or federal animal health official, or an American Fisheries Society (AFS) certified inspector or pathologist. Contact the Laboratory that will be performing the assays to coordinate sampling.

5. Sample information will include the following information at a minimum:

   - Name and address of owner
   - Location of sample collection
   - Type of water source (well, spring, surface)
   - Whether a water source is fish-free
   - Name and address of the submitting individual
   - Age, species, origin of fish (fish or eggs)
   - Number of fish present in each lot
   - Number of fish sampled in each lot
   - For *M. cerebralis* sampling, age should be given in temperature degree-days. If continuous temperature data is not available, report age in months. It should be indicated how long the fish have resided in the water supply.

B. Size/Age Groups

Environmental and species differences can markedly affect the growth rate of fish. In addition, some pathogens are most readily detected when fish are a certain size, whereas others are most readily detected when fish are a certain age. For the purpose of fish health inspections, fish are assigned to one of four groups based on either size or age depending on the pathogen of interest. The following table provides a general reference for these classifications. These classifications may not fit all species.

Table 2.2 - Suggested categories for grouping fish for sample collection

Designation	Total length	or	Age
Fry	**< 4 cm**		**0–3 months of age**
Fingerlings	**4 – 6 cm**		**4-12 months age**
Yearlings/Adults	**> 6 cm**		**Non-brood fishes greater than 12 months of age**
Broodstock	**> 6 cm**		**Sexually mature fish greater than 12 months of age and used as broodstock**

C. Lot and Inspection Definitions

Refer to Table 2.1 and to the pathogen specific sections of this Handbook for detailed information on what species of fish are susceptible to each pathogen and the conditions under which it is most readily detected.

1. **Lot of non-broodstock fish**: A group of non-brood fish of the same species and age group (see definitions of age group in Table 2.2) that have continuously shared a common water source throughout their life history. A representative sample of all strains and rearing units containing this lot shall be included.

2. **Lot of broodstock fish**: A group of sexually mature fish of the same species that share a common water source. The sample must be representative of all age groups (e.g., 3, 4, and 5 year old brood fish) and strains present at the facility.

3. **Lot Inspection**: The collection and examination of a statistically valid number of the appropriate samples from a specific lot of a susceptible species for any pathogen listed in this Handbook. Moribund fish will be included when present. Unless otherwise stated in the policies and/or regulations of the jurisdictions involved, sampling for the required pathogens will be performed at the 5% APPL with a 95% confidence level. See Table 2.3 for further explanation of the number of samples required.
    a **Exception:** In broodstock lots where there is access to ovarian fluid, sampling for the required viral pathogens will be performed at the 5% APPL with a 95% confidence level in both kidney/spleen tissues and ovarian (coelomic) fluid. Kidney/spleen and ovarian fluid samples must come from different individuals.

        ***Example*:** In a broodstock population consisting of 2500 individuals, kidney/spleen samples will be collected from 60 fish (males and/or females) and ovarian fluid will be collected from an additional 60 females for a lot inspection requiring IHNV testing.

    b **Exception**: A lot of anadromous salmon regularly monitored for *Renibacterium salmoninarum* through ELISA or quantitative PCR techniques may be considered positive for this pathogen without additional testing. Results of the monitoring must be provided to the jurisdictions involved when requested.

4. **Facility Inspection**: Lot inspection of each and every susceptible lot of fish held on the facility for any of the bacterial, viral, and parasitic pathogens listed in this Handbook.
    a **Exception:** For *Myxobolus cerebralis*, only one lot of the most susceptible species on each water source at the facility needs to be inspected. It is essential that the lot chosen has had sufficient exposure to create a detectable infection

    b **Inspection frequency:** Most regulating jurisdictions require that a history of annual inspections be submitted with the inspection report prior to permitting the importation, stocking and/or transfer of aquatic animals. It is, therefore, recommended that a program of annual facility inspections be encouraged for

any facility participating in intrastate, interstate and/ or international commerce of their animals.

D. Sample Number

Unless otherwise dictated by the receiving jurisdiction, the number of fish to be collected from each lot must be in accordance with a plan that provides 95 % confidence that at least one infected fish will be collected if the minimum assumed pathogen prevalence level (APPL) of infection equals or exceeds 5%. Examples of the number of fish to sample for various population sizes are listed in Table 2.3. Table 2.3 also includes examples of the number of fish to sample if a 2 % or 10% APPL is required by the requesting authority. If the population size is estimated to be between two grouping levels, the sample is taken from the next higher population class. (Amos, 1985; OIE, 2000; Ossiander and Wedemeyer, 1973; Thoesen, 1994).

Table 2.3 - Sample number based on an assumed pathogen prevalence level (APPL) in the population of 10%, 5 %, or 2%

Lot Size	Number of fish required for sample		
(number of fish)	10% APPL	5% APPL	2% APPL
50	20	35	50
100	23	45	75
250	25	50	110
500	26	55	130
2000	27	60	145
>100,000	30	60	150

E. Sample Collection

The order in which tissues are collected will vary depending on the tests to be run. What tissues and fluids are collected will vary depending on the size of the fish, age of the fish, purpose for which the inspection is being performed, and the requirements of the assays used by the receiving laboratory.

1. **Necropsy** – A detailed necropsy procedure can be found in "Fish Disease: Diagnosis and Treatment" (Noga, 1996)
    a. Examine and note presence of gross external lesions. If lesions are collected for histological examination, it must be done in a manner that will not compromise the aseptic collection of samples for bacteriology and virology.

    b. Collected fish are humanely euthanized immediately prior to sample collection.

> **Note**: If confirmation of *M. cerebralis* infection is to be done histologically, fish should not be killed by a blow to the head as this may compromise the integrity of skeletal elements.

c. Fry are generally only examined for viruses. Fingerling, yearling, and adult sized fish may be examined for bacteria, virus, and/or parasites.

d. The instruments used during sample collection are at a minimum cleaned between sample pools and disinfected between lots.

e. The body cavity is opened being careful not to compromise the target sample tissues with contents from the intestinal tract.

f. If it blocks access to the kidney, the swim bladder is moved.

2. **Collection of kidney cultures for the detection of *Aeromonas salmonicida, Edwardsiella ictaluri, Yersinia ruckeri and/or Renibacterium salmoninarum*. Samples for bacteriology should always be taken first with proper aseptic technique to minimize contamination.** These cultures are not usually collected from fry.
   a. A sterile swab or inoculating loop is inserted into the posterior kidney and streaked on a plate or slant (2.3.B) of the appropriate media. Streaks from up to four fish in the same lot may be made on a single culture plate. When multiple cultures will be made from one fish, reinsert the sterile inoculation loop or swab into the kidney before each plate or slant is streaked. If fish are large enough, a piece of kidney tissue may be excised and used to streak the media.
      i. *Aeromonas salmonicida, Edwardsiella ictaluri, and Yersinia ruckeri:* Brain Heart Infusion Agar (BHIA) or Trypticase Soy Agar (TSA) (2.3.A.1)

      ii. *Renibacterium salmoninarum:* SKDM-2 (2.3.A.2) if the receiving laboratory will be using bacterial culture for the confirmation of *R. salmoninarum*

   b. Sample any organs with visible lesions.

   c. Incubate media and identify pathogens by the methods described in Chapter 3.

   d. A smear for the *R. salmoninarum* FAT (3.5.A) is made from the posterior kidney on a microscope slide. The slides will be screened by FAT as described in 3.5.A.

   e. If the polymerase chain reaction technique (PCR) (3.5.B.2) will be used to confirm a positive *R. salmoninarum* FAT slide, a kidney sample is collected after sampling of the kidney is completed for bacteriology and virology.

      Note: Approximately 25 mg of kidney is collected into a sterile vial and frozen. Tissues collected for PCR archiving should be labeled so that those tissues can be identified

individually if corresponding FAT slides are found positive for *R. salmoninarum*.

f. Ovarian fluid, when available, may be collected from spawning female broodstock for detection of *R. salmoninarum*. The ovarian fluid sample may be obtained from an aliquot of the sample collected for viral analysis (2.2.E.3.e) and processed at the laboratory as described in 3.5.A.1.b

3. **Collection of tissues for the detection of viral agents**. During collection, transport, and storage prior to processing, samples should be kept chilled (0-4°C). **Do not freeze.** During processing, samples should be kept on ice and at no time exceed 15°C or virus viability may be compromised. Tissues for viral testing may be collected and stored in a viral transport media such as Hank's Buffered Salt Solution (HBSS) with or without antibiotics (2.3.C.2). The pH should be maintained within the 7.2 - 7.6 range. The samples must be processed and inoculated onto cell cultures within 72 hours of collection and 48 hours or less is recommended.
    a. From fry, the entire fish is collected and placed into sterile containers; when present, yolk sacs should be removed to reduce toxicity in cell culture and muscle tissue may be trimmed off as needed to maintain a reasonable sample volume.

    b. From fingerling-sized fish, the visceral mass including the kidney is collected and placed into sterile containers. If the stomach is filled with feed, it may be removed.

    c. From yearling/adult fish, approximately equal amounts of the spleen and kidney are obtained using aseptic technique and placed into sterile containers. Tissues from up to 5 fish may be pooled in the same container with approximately an equal amount of tissue from each fish. Total sample volume should not exceed 1.5 grams of tissue.

    d. From broodstock fish, approximately equal amounts of the spleen and kidney are obtained using aseptic technique and placed into sterile containers. Tissues from up to 5 fish may be pooled in the same container with approximately an equal amount of tissue from each fish. Total sample volume should not exceed 1.5 grams of tissue.

    e. From female broodstock at spawning, ovarian fluid is collected into an appropriately sized sterile container. Approximately equal volumes (1 ml per fish) of ovarian fluid from up to 5 fish may be pooled in the same container.

4. **Collection of specimens for the detection of *Myxobolus cerebralis* evaluation**. Selection of appropriate species and age should be made using Table 1 and information in 5.2. (Lorz and Amandi 1994; Meyers 1997)
    a. Fish from the same lot may be processed in pools of up to 5 fish by pepsin-trypsin digest (PTD). For confirmation by PCR or histology, head/core samples

are processed individually. Therefore, in order to track positive pools, all corresponding samples must be labeled appropriately.

b. From fingerling and yearling fish (less than 15 cm), the entire head, including opercles, is severed from the body.

c. For larger fish where size makes collecting the entire head impractical, a wedge or core samples may be taken. Include gill arches for more resistant species (5.2).
   i. A triangle-shaped wedge is cut posterior to the orbit at the dorsal surface almost to the ventral edge of the opercula. The top (dorsal) portion of the wedge should measure 1.5 cm (Figure 2.1).

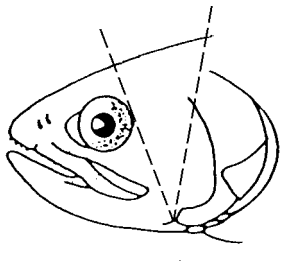


Figure 2.1. Diagram of location of wedge sample from adult fish.

   ii. A core sample is taken by inserting a biopsy or boring tool (at least 19mm diameter; boring drill bit or sharpened pipe fitted to a drill work well) into the dorsal surface of the head just posterior to the eyes and forcing it ventrally until it penetrates into the mouth. (Figure 2.2)

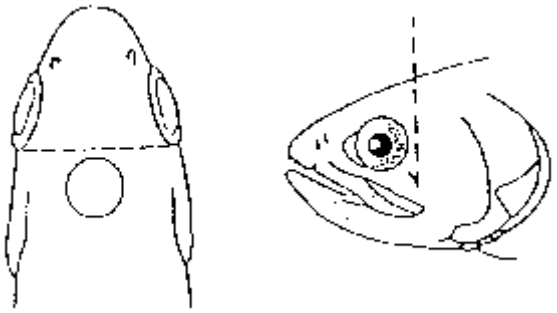


Figure 2.2. Diagram of location of core sample from adult fish.

d. Each head, wedge or core sample should be split such that each piece contains all the tissue layers. The tissue that is to be processed by PTD for screening should be placed in a plastic bag and transported to the laboratory on ice. Tissues representing up to 5 fish may be combined for PTD assay.

e. The other half-head, wedge or core is preserved in a manner suitable for confirmatory testing as follows:
    i. For PCR confirmation, at the time of collection, refrigerate, place on ice or freeze. Upon receipt by the laboratory, samples may be frozen at –20°C.

    Note: extreme care should be taken in the collection of samples in which PCR confirmation may be used. Refer to Chapter 6 for appropriate precautions.

    ii. For histologic confirmation, at the time of collection place tissue in a fixative suitable for histology, such as 10% neutral buffered formalin (2.3.C.3) or Davidson's (2.3.C.4) fixative. Use a 10:1 (volume/volume) volume of fixative to sample.

f. Number individual samples to correspond to the tissue pool to be analyzed by PTD.

5. **Collection of tissue for the detection of *Ceratomyxa shasta*** (Bartholomew 2001)
    a. For detection of *C. shasta,* fish are sampled individually and tissues are not pooled for examination.

    b. Wet mounts are prepared from intestinal scrapings from the posterior intestine and from any lesions. When possible, fish should be examined immediately after death, but whole fish or intestines can be shipped on ice and examined within 24 h.

    c. For PCR confirmation of *C. shasta*, excise a small portion of the lower intestine (about 2-5 mm) and transfer to a vial with 500 µl DNA extraction buffer (5.6.G). Alternatively, the sample may be frozen or fixed in 100% ethanol (EtOH). Samples in ethanol may be stored at room temperature; those in extraction buffer should be refrigerated or frozen for long-term storage.

    Note: extreme care should be taken in the collection of samples in which PCR confirmation may be used. Refer to Chapter 6 for appropriate precautions.

6. **Collection of tissue for the detection of *Bothriocephalus acheilognathi***
    a. Collected fish are best processed shortly after euthanasia, but may be transported on ice for up to 24 h. Fixed specimens are not acceptable because of recovery and identification problems. This is especially true for small tapeworms.

    b. If fish larger than 20 cm are to be examined, the anterior third of intestinal tracts can be removed and placed in bags on ice to avoid transporting whole large fish.

c. To remove the intestine, cut at the anus and just posterior to the stomach. Unravel intestines gently with fingers and cut off the anterior third and place in bag on ice. Discard the lower two-thirds of the intestines.

d. Fish smaller than 20 cm are best transported to the laboratory alive, but may be shipped whole on ice in plastic bags.

7. **Collection of sample for the detection of *Tetracapsula bryosalmonae*.** (Klontz and Chacko 1983; Hedrick et al. 1986; Kent 1994)

a. For *T. bryosamonae* fish are sampled individually and tissues are not pooled for examination. However, impressions of more that one fish can be made on a single microscope slide.

b. Impressions of kidney tissue of each fish are made by excising a small ($5mm^2$) piece of tissue, blotting the tissue on paper to remove the excess blood, and making serial impressions on an alcohol-cleaned slide. Tissue is fixed in either absolute (100%) methanol (MeOH) for 5 min for Leishman-Giemsa staining, or, in acetone-ethanol (60:40) at –20°C for 10 min for lectin staining. The slide is labeled appropriately (location, reference), and stored in a slide box. For prolonged storage of slides for lectin staining, they should be stored dessicated at –70°C.

c. A small piece of kidney from each fish is saved in 10% neutral buffered formalin (2.3.C.3) or other suitable tissue fixative (Davidson's (2.3.C.4)) for confirmation of the presence of *T. bryosalmonae* by histopathology.

2.3 Reagents, Media, and Media Preparation

A. Growth Media – Most of these media are commercially available as pre-made formulas or as bases, which can be easily made in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations.

1. **Brain Heart Infusion Agar (BHIA) and Tryptic Soy Agar (TSA)** – These two basic agars are interchangeable for bacterial cultures obtained during an inspection. They are both commercially available.

2. **Selective Kidney Disease Medium-2 (SKDM-2)** (Austin, et.al.1983)- for selective isolation of *Renibacterium salmoninarum*

Peptone	10 gm
Yeast Extract	0.5 gm
L-Cysteine HCL	1 gm
Agar	15 gm
Distilled Water	to 1000 ml

Adjust pH to 6.5 before adding agar. Autoclave for 15 minutes at 121°C.
Cool to ~ 50°C and add:

Fetal Bovine Serum 200.0 mls

The following antibiotics can also be added to the SKDM-2 to reduce overgrowth from other bacterial organisms (Austin, et. al. 1983).

4.0 mls Cyclohexamide (1.2 gms Cyclohexamide in 96 mls dH_2O)
1.0 ml D-Cycloserine (0.3 gms D-Cycloserine in 24 mls of dH_2O)
2.0 mls Polymyxin B-sulfate (0.3 gms Polymyxin B-sulfate in 24 mls of distilled H_2O)
1.0 mls Oxolinic Acid (0.06 gms Oxolinic Acid in 24 mls of 5% NaOH)

B. Media Preparation

1. **Plate Media -**
   a. Prepare media in stainless steel beakers or clean glassware according to manufacturer instructions. Check pH and adjust if necessary. Media must be boiled for one minute to completely suspend agar. Use of a stir bar will facilitate mixing of agar.

   b. Cover beaker with foil, or pour into clean bottles being sure to leave lids loose. Sterilize according to manufacturer's instructions when given, or at 121°C for 15 minutes at 15 pounds pressure.

c. Cool media to 50°C.

d. Alternatively, media can be autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with media type, date and initials. When media is needed, boil, microwave or use a water bath to completely melt the agar. Cool to 50°C. Avoid reheating media multiple times before use.

e. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface. Aseptically mix any antibiotic solutions, sheep's blood, or Fetal Bovine Serum into the media at this temperature.

f. Label the bottom of each plate with medium type and date prepared.

g. Remove bottle cap and pour plates or dispense with a sterile Cornwall pipette, lifting each petri dish lid as you go. Pour approximately 15 to 20 ml per petri dish. Replace lids as soon as the plate is poured.

h. Immediately wash medium bottle, cap, and pipette in hot water to remove agar and clean up any spilled agar.

i. Invert plates when the media has cooled completely (~ 30-60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid.

j. Allow plates to sit overnight at room temperature. Store plates upside down in the refrigerator in a tightly sealed plastic bag or plate storage tin.

k. Follow manufacturer's recommendation for storage period of prepared media. Each batch should be labeled with date of preparation and/or an expiration date.

2. Tube Media

a. Prepare media in stainless steel beakers or clean glassware according to manufacturer's instructions. If the medium contains agar, boil for one minute to completely suspend the agar. Use of a stir bar will facilitate mixing of agar.

b. Media with indicators must be pH adjusted. This can be done when medium is at room temperature; otherwise compensation for temperature needs to be made.

c. Arrange test tubes in racks. Disposable screw cap tubes can be used for all tube media.

d. Use an automatic pipetter or Pipette-aid™ to dispense the medium. If using the Brewer or Cornwall pipette, prime with deionized water and then pump the water out of the syringe prior to pipetting. Discard the first few tubes of media

that are dispensed. Dispense approximately 5 to 10 ml media in 16x125mm or 20x125mm tubes. Close caps loosely.

e. Immediately after use, rinse the automatic pipetter in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.

f. Follow manufacturer's recommendation for autoclave time and temperature.

g. If making slants, put tubes in slant racks after autoclaving. Adjust the slant angle to achieve the desired slant angle and butt length (i.e. long butt and short slant for TSI or a standard slant over ¾ of the tube length for TSA or BHIA).

h. Cool completely to room temperature in the slanted position. Tighten caps.

i. Label the tubes or the tube rack with type of medium and date made.

j. Store at 2-8°C, following manufacturer's recommendation for long-term storage.

C. Reagents

1. 70% Ethanol (EtOH)

Ethanol (95%)	737 ml
Distilled Water	to 1000 ml

2. Hanks Balanced Salt Solution (HBSS)

10X HBSS	100.0 ml
Cell Culture Grade Water	895.0 ml
$NaHCO_3$ (7.5%)	5.0 ml

If antibiotics are used, subtract 320 mls of water and add in its place

Penicillin/Streptomycin (16%)	160.0 ml
Penicillin G (10,000 units/ml)	
Streptomycin sulfate (10,000 ug/ml)	
Fungizone	160.0 ml
250 ug/ml Amphotericin B	
205 ug/ml desoxycholate	

Mix. Filter with 0.22 um filter. Store at 4° C.

3. 10% neutral buffered formalin (10% NBF)

Formalin (37% Formaldehyde)	100 ml
Distilled Water	900 ml
Sodium Phosphate (monobasic)	4 g
Sodium Phosphate (dibasic)	6.5 g

Store at room temperature.

4. Davidson's Fixative

95% Ethanol	600 ml
Formalin (37% Formaldehyde)	400 ml
Acetic Acid (Glacial)	200 ml
Distilled Water	600 ml

Store at room temperature.

2.4 Glossary

Anterior kidney - portion of the kidney containing hematopoietic tissues with little or no urinary function. Generally it is the portion of the kidney closest to the head.

Aseptic technique – prevention of contact with microorganisms not contained in the target tissue.

Disinfect – To free from pathogenic organisms or render those organisms non-infectious.

ELISA – Enzyme-linked immunosorbent assay

Inspector - a federally accredited veterinarian, a state or federal animal health official, or an American Fisheries Society (AFS) certified inspector or pathologist.

Health history – past events pertaining to infectious or non infectious agents found during routine monitoring or diagnostic testing.

Life history –all life stages including eggs

PCR – Polymerase chain reaction

Posterior kidney - portion of the kidney containing excretory elements and responsible for performing urinary function.

Statistically valid number – the number of tissue or fluid samples sufficient to assess the risk of a particular pathogen being present in the population of interest. Selection of this sample number involves specifying both a minimum assumed prevalence level of the pathogen in the population and the minimum acceptable confidence interval for the detection of that pathogen.

Sterile – free from living microorganisms.

Strain – A fish population that exhibits reproducible physiological, morphological, or cultural performance characteristics that are significantly different from other fish populations or a broodstock derived from such a population and maintained therafter as a pure breeding population.

Strain resistance – the development of disease resistance by a particular strain of fish either through natural or artificial selection.

Susceptible species – any species capable of becoming infected with a particular pathogenic organism.

Therapies – the use of any drugs and/or chemicals for the treatment of disease.

Visceral mass – contents of the abdomen of the fish that includes the liver, spleen, stomach, pyloric caeca, intestine, and kidney.

Water source - a spring, lake, river, stream, creek, or aquifer. Multiple wells from the same aquifer or multiple inlets from the same surface water supply constitute a single water source for a facility. Wells in different aquifers or with demonstrably different water chemistry must be considered different water supplies even if present on a single facility. Unless they are known to come from the same aquifer, every well should be considered a separate source.

2.5 References

Austin, B., T.M. Embley, and M. Goodfellow (1983) Selective isolation of *Renibacterium salmoninarum*. FEMS Microbiol. Letters 17:111-114

Bartholomew, J. L. 2001. Salmonid ceratomyxosis. *in*: J Thoesen, ed. Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. Blue Book 4th Edition. Fish Health Section, American Fisheries Society.

Difco Manual, 11th Ed. (1998) DIFCO Laboratories, Division of Becton Dickinson & Co., Sparks, Md. (This manual is also available on the internet at: http://www.bd.com/industrial/difco/DifcoManual.pdf)

Hauck, K. and S. Landin. 1997. Detection of the Whirling Disease Agent (*Myxobolus cerebralis*) *in:* T. R. Meyers, ed. Fish Parasitology Section Laboratory Manual. Alaska Fish and Game. Special Publication No. 12.

Hedrick, R. P., M. L. Kent and C. E. Smith. 1986. Proliferative Kidney Disease in Salmonid Fishes. US Fish and Wildlife Service Fish Disease Leaflet 74

Kent, M. L. 1994. Proliferative Kidney Disease *in*: ed. J Thoesen, Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. Blue Book 4th Edition. Fish Health Section, American Fisheries Society.

Kincaid, H.L. 1981. Trout Strain Registry. Published by USFWS, National Fisheries Center, Leetown, WV

Klontz, G. W. and A. J. Chacko, 1983. Methods to detect the organism causing proliferative kidney disease in salmonids. Bulletin of the European Association of Fish Pathologists 3:33-36.

Lorz, H. V. and A. Amandi. 1994. Whirling Disease of Salmonids *in*: Edited by J. Thoesen, Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. Blue Book 4th Edition. Fish Health Section, American Fisheries Society.

National Fish Strain Registry. 2001. The following website is sponsored and maintained by USGS, Northern Appalachian Research Laboratory and USFWS, Division of Fish Hatcheries: http://159.189.37.201/

Noga, E. J., 1996. Fish Disease: Diagnosis and Treatment. Mosby Year Book Inc., St. Louis, Missouri. 367 pp.

Ossiander, F. J., and G. A. Wedemeyer, 1973. Computer program for sample size required to determine disease incidence in fish populations. J. Fish. Res. Board Can., 30:1383-1384

Chapter 3

Bacteriology

3.1 Introduction

The following chapter describes inspection procedures for bacterial pathogens of fish that may be required for a fish health inspection. The target bacterial species include *Aeromonas salmonicida*, *Yersinia ruckeri*, *Edwardsiella ictaluri* and *Renibacterium salmoninarum*. Chapter 2 describes procedures for proper sampling of fish tissues to ensure detection of any of these pathogens during a fish health inspection.

Presumptive identifications of *A. salmonicida (*subspecies *salmonicida and achromogenes)* (3.2)*, E. ictaluri* (3.4)*,* and *Y. ruckeri* (3.3) are based on Gram staining properties, and characteristic biochemical reactions. Confirmatory identification consists of fluorescent antibody testing using fluorescein-conjugated, species-specific antibody (3.7.E). Known isolates of *A. salmonicida, E. ictaluri* and *Y. ruckeri* are purchased from ATCC and are used as positive controls. Single, unknown isolates may be used to test for all three of these organisms.

The presumptive identification of the Gram-positive bacterium *R. salmoninarum* (3.5) is based upon serological methods. For purposes of initial screening and detection of the pathogen, the direct fluorescent antibody technique (FAT) on kidney smears and ovarian fluid samples employed (3.7.E). Documentation exists which indicates the possibility for false positive results caused by bacterial organisms which cross react with antibodies prepared against *R. salmoninarum* (Austin, 1985; Bullock, 1980; Brown, et al, 1995). For this reason, it is important to follow steps described below to confirm that a positive FAT result is due to the presence of this pathogen. **Exception: A**nadromous salmonids regularly monitored for *R. salmoninarum* with ELISA or quantitative PCR techniques may be considered positive without additional testing by FAT.

Any FAT results which appear positive for *R. salmoninarum* should be confirmed by either culture of kidney tissue on selective kidney disease medium (SKDM-2) (3.5.B.1), or by testing the positive tissues with the polymerase chain reaction (PCR) technique (3.5.B.2).

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U.S. Fish and Wildlife Service, the United States government, and/or the American Fisheries Society. Any comparable instrument, laboratory supply or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

3.2 *Aeromonas salmonicida (Furunculosis)*

Aeromonas salmonicida may be difficult to detect based on a variety of environmental, physiological and host factors. At water temperatures between 14-24^{o}C, fish can develop clinical disease within 4-12 days after bacterial exposure in the water supply. At temperatures below 13^{o}C, chronic and latent infections are more likely to develop. This pathogen has been associated with disease in a variety of salmonid and non-salmonid species. It is generally accepted that any freshwater fish can carry the bacteria with or without showing signs of disease (Thoesen, 1994, Bullock, et al. 1983). The typical form of the bacterium (subspecies *salmonicida*) produces a soluble brown pigment on tryptic soy agar after two to three days of growth at 20^{o}C. The less common and atypical strain of this pathogen (subspecies *achromogene*s) does not produce pigmentation under these incubation conditions. Both strains, however, have been associated with disease in a variety of fish species (Paterson et al. 1980).

A. Summary of Screening Test

Bacterial Culture and Biochemical Analysis

1. Aseptically inoculate samples into TSA tubes or onto plates as described in Chapter 2

2. Incubate for 24-48 hours at 20 - 24°C (room temperature). If no growth occurs at either 24 or 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are reported as negative for *A. salmonicida***.

3. When primary culture occurs on tubes or plates use a sterile loop or needle to select a single, isolated colony to subculture onto fresh TSA plates. If colonies are not well isolated on the original media, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.

4. Incubate at 20-24°C for 24 hours to allow bacterial growth; all tests should be performed on 24-48 hour cultures.

5. Using a sterile needle or small loop, pick individual distinct bacterial colonies representing each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each isolated colony and record all colony characteristics on the data sheet.

6. Begin initial identification of pure strain bacterial cultures (3.A1):
    a. Colony Pigmentation: Typical strains of *A. salmonicida* have brown diffusible pigment after 2-3 days of incubation. Some strains may not be pigmented (subsp. *achromogene*s).

    b. Gram Determination (3.7.A): *A. salmonicida* is Gram Negative. Gram Positive isolates may be reported as negative for *A. salmonicida*.

    c. Presence of Cytochrome Oxidase (CO) (3.7.B): Most are CO positive. Rarely, CO negative strains of *A. salmonicida* subsp. *salmonicida* have been encountered (Chapman, et al. 1991).

d. Motility (3.7.C): *A. salmonicida* is non-motile. Motile isolates may be reported as negative for *A. salmonicida*.

7. Perform biochemical testing on each isolate (3.A1):
    a. Tube Method (3.7.D.1)
        i. Glucose fermentation (3.7.D.1.a) using OF basal medium containing glucose will produce an Oxidative/fermentative (O/F) or a Fermentative (F) result with Gas (most strains produce gas, but some may be weak or variable in this production). **Any isolate with a result other than this may be reported as negative for *A. salmonicida*.**

        ii. Gelatinase (3.7.D.1.c) and Indole (3.7.D.1.d) are considered together with the pigmentation of the isolate for interpretation of the results.
            1. Brown diffusible pigmented isolates that are Gelatinase positive and Indole negative are **PRESUMPTIVELY positive** *A. salmonicida* sub-species *salmonicida*.

            2. Non-pigmented isolates that are Gelatinase negative and Indole positive are **PRESUMPTIVELY positive** *A. salmonicida* sub-species *achromogens.*

            3. **Isolates yielding any other combination of these results may be reported as negative for *A. salmonicida***.

    b. Commercial Identification Systems (3.7.D.2)
        i. Biolog (3.7.D.2.b)

        ii. API: If isolates are tested with the commercial system API described in Section 3.7.D.2.a, it is recommended that the Reference profiles be consulted in 3.A2.

8. When testing is complete, either cryopreserve isolates of interest, or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.

9. Positive control isolates of *Aeromonas salmonicida* can be obtained from the American Type Culture collection (ATCC). The internet location for ATCC is http://www.atcc.org. Below are suggested isolates to use for positive control cultures:
    a. *A.salmonicida* subspecies s*almonicida* – ATCC # 14174

    b.*A.salmonicida* subspecies *achromogenes* – ATCC # 10801

    c. A **cytochrome oxidase negative** isolate is also available – ATCC # 49385

B. Confirmatory Test

Fluorescent Antibody Test (FAT) (3.7.E) is performed on at least one representative isolate from each lot inspected and found positive during screening. Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control. A list of sources from which antibodies may be obtained is provided in 3.7.E.

3.3 *Yersinia ruckeri (Enteric Redmouth Disease, ERM)*

Yersinia ruckeri affects fingerlings of potentially all salmonids. In salmonids, mortality increases dramatically following periods of stress due to environmental conditions and handling. Survivors of enteric redmouth disease outbreaks become carriers, after which the bacteria are shed from the intestinal tract in large numbers during a regular 36 to 40 day cycle. Severity of infection decreases at water temperatures below 10°C. *Y. ruckeri* has been isolated from the kidney of some non-salmonids as well. It is generally accepted that, although not ubiquitous in the environment, any freshwater fish can carry the bacteria with or without exhibiting signs of disease (Thoesen ,1994; Bullock, 1984).

A. Summary of Screening Test

Bacterial Culture and Biochemical Analysis

1. Aseptically inoculate samples onto tubes or plates as described in Chapter 2

2. Incubate for 24-48 hours at 20 - 24°C, (room temperature). If no growth occurs at either 24 or 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are discarded and reported as negative for *Y. ruckeri***.

3. When primary culture occurs on tubes or plates use a sterile loop or needle to select a single isolated colony to subculture onto fresh TSA or BHIA plates. If colonies are not well isolated on the original media, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.

4. Incubate at 20 - 24°C for 24 hours to allow bacterial growth; all tests should be performed on 24-48 hour cultures.

5. Using a sterile needle or small loop, pick individual distinct bacterial colonies to represent each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.

6. Begin initial identification of pure strain bacterial cultures (3.A1):
    a. Gram Determination (3.7.A): *Y. ruckeri* is Gram Negative. **Gram Positive isolates may be reported as negative for *Y. ruckeri*.**

    b. Presence of Cytochrome Oxidase (CO) (3.7.B): *Y. ruckeri* is CO negative. **CO positive isolates may be reported negative for *Y. ruckeri***.

    c. Motility (3.7.C): *Y. ruckeri* is motile. **Non-Motile isolates may be reported as negative for *Y. ruckeri***.

7. Perform biochemical testing on each isolate (3.A1):
    a. Tube Method (3.7.D.1)
        i. Triple Sugar Iron (TSI) (3.7.D.1.b): *Y. ruckeri* will yield an Alkaline over Acid (K/A) or Alkaline over Acid with Gas (K/AG) result. **Any**

isolate with a result other than this may be reported as negative for *Y. ruckeri.*

ii. Carbohydrate Utilization (3.7.D.1.e):
   1. Arabinose cannot be utilized (fermented) by *Y. ruckeri*.

   2. Rhamnose cannot be utilized (fermented) by *Y. ruckeri*.

   3. Sucrose cannot be utilized (fermented) by *Y. ruckeri*.

   4. Salicin cannot be utilized (fermented) by *Y. ruckeri*.

   5. **Isolates yielding positive results for any of these tests may reported as negative for *Y. ruckeri***.

iii. Malonate Test (3.7.D.1.g): **Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri***.

iv. Indole Test (3.7.D.1.d): **Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri***.

v. Esculin Test (3.7.D.1.h): **Isolates yielding positive results for this test may be reported as negative for *Y. ruckeri***.

vi. Decarboxylase Test (Lysine) (3.7.D.1.f): **Isolates yielding negative results for this test may be reported as negative for *Y. ruckeri.***

vii. Sorbitol (3.7.D.1.e):
   1. Isolates that satisfy all previous conditions in this section are **PRESUMPTIVELY positive** for *Y. ruckeri*.

   2. Sorbitol is used to differentiate between Type I and Type II *Y. ruckeri*.
      a. Isolates yielding negative results for this test are **PRESUMPTIVELY positive** for *Y. ruckeri* Type I.

      b. Isolates yielding positive results for this test are **PRESUMPTIVELY positive** for *Y. ruckeri* Type II.

b. Commercial Identification Systems (3.7.D.2)
   i. Biolog (3.7.D.2.b)

   ii. API: If isolates are tested with the commercial system API described in Section 3.7.D.2.a, it is recommended that the Reference profiles be consulted in 3.A2.

8. When testing is complete, either cryopreserve isolates of interest, or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.

9. Positive control isolates of *Yersinia ruckeri* can be obtained from the American Type Culture collection (ATCC). The internet location for ATCC is http://www.atcc.org. Below are suggested isolates to use for positive control cultures:
    a. *Yersinia ruckeri* serovar Type I – ATCC # 29473

    b. *Y. ruckeri* Type II – ATCC # 29908

B. Confirmatory Test

Fluorescent Antibody Test (FAT) (3.7.E) is performed on at least one representative isolate from each lot inspected and found positive during screening.

1 A separate antibody must be utilized for confirmation of either Type I or II strains.

2 Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control.

3 A list of sources from which antibodies may be obtained is provided in 3.7.E.

3.4 *Edwardsiella ictaluri (Enteric Septicemia of Catfish, ESC)*

The primary host species of this pathogen include all species of catfish, tilapia, as well as other warm water species. Both fingerlings and adults can be affected by the disease. In catfish, the bacterium is transmitted through the olfactory system to the brain, where the typical "hole in the head" lesions can be observed during an *E. ictaluri* epizootic. Disease typically occurs at water temperatures between 20°C and 30°C. Experimental infections have been established in salmonids. It is generally accepted that any warm water species of fish can carry this pathogen with or without exhibiting signs of disease (Austin & Austin, 1987; Thoesen, 1994, Bullock & Herman, 1985; Hawke et al, 1981).

A. Summary of Screening Test

Bacterial Culture and Biochemical Analysis

1 Aseptically inoculate samples onto tubes or plates as described in Chapter 2.

2 Incubate for 24-48 hours at 28 - 30°C. Alternatively this organism can be grown at 20 - 24°C (Plumb et al, 1989). If no growth occurs at 24 and 48 hours, record this information on the data sheet. **If no growth occurs after 96 hours, samples are discarded and reported as negative for *E. ictaluri***.

3 When primary culture occurs on tubes or plates use a sterile loop or needle to select a single colony to subculture onto fresh TSA or BHIA plates. If colonies are not well isolated, a new plate will have to be streaked over the entire plate surface to achieve isolation of bacteria.

4 Incubate at temperature used above for 24 hours to allow bacterial growth; all tests should be performed on 24-48 hour cultures.

5 Using a sterile needle or small loop, pick individual distinct bacterial colonies to represent each colony type. Use of a dissecting microscope can aid in distinguishing between differing colony types. Assign an isolate number to each pure colony and record all colony characteristics on the data sheet.

6 Begin initial identification of pure strain bacterial cultures (3.A1):
   a. Gram Determination (3.7.A): *E. ictaluri* is Gram Negative. **Gram Positive isolates may be reported as negative for *E. ictaluri***.

   b. Presence of Cytochrome Oxidase (CO) (3.7.B): *E. ictaluri* is CO negative. **CO positive isolates may be reported negative for *E. ictaluri***.

7 Perform biochemical testing on each isolate (3.A1):
   a. Tube Method (3.7.D.1)
      i. Triple Sugar Iron (TSI) (3.7.D.1.b): *E. ictaluri* will yield an Alkaline over Acid (K/A) or Alkaline over Acid with Gas (K/AG) result. **Any**

isolate with a result other than this may be reported as negative for *E. ictaluri*.

ii. Carbohydrate Utilization (3.7.D.1.e):

1. The following carbohydrates cannot be utilized (fermented) by *E. ictaluri*:

Arabinose
Rhamnose
Sucrose
Salicin

2. **Isolates yielding positive results for any of these tests may be reported as negative for *E. ictaluri***.

iii. Malonate Test (3.7.D.1.g): **Isolates yielding positive results for this test may be reported as negative for *E. ictaluri***.

iv. Indole Test (3.7.D.1.d): **Isolates yielding positive results for this test may be reported as negative for *E. ictaluri***.

v. Esculin Test (3.7.D.1.h): **Isolates yielding positive results for this test may be reported as negative for *E. ictaluri***.

vi. Decarboxylase Test (Lysine) (3.7.D.1.f): **Isolates yielding negative results for this test may be reported as negative for *E. ictaluri***.

vii. Manitol Utilization Test (3.7.D.1.e):

1. **Isolates yielding positive results for this test may be reported as negative for *E. ictaluri***.

2. Isolates yielding negative results for this test and that satisfy all previous conditions in this section are **PRESUMPTIVELY positive** for *E. ictaluri*

b. Commercial Identification Systems (3.7.D.2)

i. Biolog (3.7.D.2.b)

ii. API: If isolates are tested with the commercial system API described in Section 3.7.D.2.a, it is recommended that the Reference profiles be consulted in 3.A2.

10 When testing is complete, either cryopreserve isolates of interest, or discard bacterial plates and biochemical tubes in a biohazard bag and autoclave before proper disposal.

11. Positive control isolates of *Edwardsiella ictaluri* can be obtained from the American Type Culture collection (ATCC). The Internet location for ATCC is http://www.atcc.org.

B. Summary of Confirmatory Test

Fluorescent Antibody Test (FAT) (3.7.E) is performed on at least one representative isolate from each inspection. Positive bacterial isolates will fluoresce strongly and have the same morphology as the positive control. A list of sources from which antibodies may be obtained is provided in 3.7.E.

3.5 *Renibacterium salmoninarum (Bacterial Kidney Disease, BKD)*

Renibacterium salmoninarum infections can occur at any life stage in salmonid populations. Clinical signs of disease are uncommon in fish less than 6 months of age. Mortality has been reported at water temperatures between 4°C and 20.5°C, with the disease progressing rapidly between 15°C to 20.5°C. Acute and sub-acute forms of disease are less common than the more typical chronic form of disease, characterized internally by a large edematous kidney that appears gray and corrugated (Thoesen, 1994; Bullock & Herman, 1988; Austin, 1987).

A. Summary of Screening Test

Fluorescent Antibody Test (FAT) (3.7.E)

Collect tissues as described in Chapter 2 and prepare FAT slides (**Exception: A**nadromous salmonids regularly monitored for *R. salmoninarum* with ELISA, quantitative PCR, or MFAT techniques may be considered positive without additional testing by FAT).

1. Collect tissues as described in Chapter 2 and prepare FAT slides:
    a. Kidney – prepare kidney smear on a non-coated or acetone-cleaned glass slide
        i. Place a piece of posterior kidney or homogenized preparation on the slide.

        ii. Create a thin smear on the surface of the glass slide.

    b. Ovarian Fluid pellet smear:
        i. After pooled ovarian fluid samples are processed and the appropriate amount of supernatant removed for virology assays (see 4.4.C), the pellet is re-suspended in the remaining ovarian fluid by thorough vortexing or repeat pipetting.

        ii. Transfer two 1.5 mL aliquots from each original pool of ovarian fluid (or 0.5 mL per fish) to sterile, labeled 1.5 mL microcentrifuge tubes (see **Note**). Freeze the remainder of the sample at -20°C for PCR confirmation.

        iii. Centrifuge the 1.5 mL aliquots at 10,000 X g for 15 minutes (see **Note**).

        iv. The pellet is carefully removed with a small amount of supernatant using a sterile pipette and a thin smear is prepared on a glass slide. Pellets originating from the same ovarian fluid sample tube may be combined on one slide.

        Note: Elliot and McKibben (1997) document the importance of using a minimum of 10,000 X g for sufficient sedimentation of *R. salmoninarum* from 0.5 mL of ovarian fluid collected from individual fish. Enough ovarian fluid must be transferred from each pool to represent 0.5 mL for each fish represented in the pool (i.e.2.5 mLs from a 5-fish-

pool). Most 15 mL centrifuge tubes used for collection and processing of ovarian fluid samples cannot be centrifuged at a relative centrifugal force (rcf) as high as 10,000 X g. It is, therefore, necessary to aliquot the appropriate amount of fluid into microcentrifuge tubes suitable for the required rcf.

2. After the tissue smear is heat fixed or air dried, slides are fixed in acetone for 5 minutes.

3. Stain slides with FITC-conjugated *R.salmoninarum* antisera as described in 3.7.E.2.

4. Examine at least 50 fields using oil immersion at 1000X magnification to detect the 1.0 X 0.5 micron bacterial cells, which should appear stained as an "apple green" fluorescence.
    a. **Smears which do not show any fluorescent bacterial cells may be discarded and reported as negative for *R. salmoninarum*.**

    b. Any smears, which have "apple green" fluorescent, diplo-bacilli bacterial cells present measuring approximately 1.0 X 0.5 microns, shall be considered **PRESUMPTIVELY positive** for *R. salmoninarum.*

5. It is preferable to use tissue infected with *R.salmoninarum* for a positive FAT control. Positive control culture isolates of *Renibacterium salmoninarum,* however, can be obtained from the American Type Culture collection (ATCC). The internet location for ATCC is http://www.atcc.org. Suppliers of commercially prepared antibodies for FAT may also provide positive control materials for use in this assay (see 3.7.E source list).

B. Confirmatory Tests

1 **Bacterial Culture (**Austin et al,1983)
    a. At the time samples are collected during the inspection (2.2.E.2), aseptically inoculate samples of tissues onto plates containing selective kidney disease media (SKDM-2) (3.6.A.3).

    b. Incubate for 2-3 weeks at 15^{o}C in a humid chamber to prevent dehydration of media.

    c. At 2-3 weeks, observe plates for growth of pinpoint bacterial colonies.
        i. If *R. salmoninarum* is presumptively identified in FAT, corresponding samples inoculated onto SKDM-2 should be examined weekly.

        ii. If no growth, continue to incubate plates for up to 6 weeks, and examine them several times per week for growth.

iii. **If no growth after 6 weeks samples may be discarded and reported as negative for *R. salmoninarum*.**

iv. If growth of small (2 mm diam.), smooth, white round colonies is observed, obtain inoculum from colony and confirm identification using FAT or PCR.

1. **If FAT or PCR results on culture are positive sample is reported as positive for *R. salmoninarum*.**

2. **If FAT or PCR results on culture are negative sample is reported as negative for *R. salmoninarum***

Note: The slow growth of this organism makes phenotypic characterization of suspect isolates difficult and time consuming. The inspector may consider pursuing phenotypic characterization if the detection of *R.salmoninarum* by these techniques continues to be questionable (consult Austin and Austin, 1993).

2. Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA

The Polymerase Chain Reaction technique employs oligonucleotide primers to amplify segments of the gene that codes for the 57 kDa protein of *R. salmoninarum* (Chase and Pascho, 1998). DNA is extracted from fish tissues and amplified initially with forward and reverse primers. The amplified DNA product is then re-amplified using a "nested PCR" technique. The DNA products from both amplifications are then visualized by agarose gel electrophoresis. The following procedures have been adapted from those of Chase and Pascho (1998), and have been reviewed and approved by the authors.

a. Extraction of DNA from Kidney and Ovarian Tissues (**NOTE**: The following procedure employs an extraction kit available from Qiagen, Inc. (http://www.qiagen.com). DNA Extraction kits of similar efficacy are available from many other sources, and can be utilized as alternatives for extraction of DNA in this protocol. These kits utilize "spin columns" for binding and elution of DNA from tissue lysates. Most do not require the use of highly toxic reagents and reduce the chance of contamination during extraction).

i. Procedures:

1. Transfer 25-50 mg of kidney tissue, or 50µl ovarian fluid, into a 1.5 ml Micro centrifuge tube. Tissue can be fresh or previously frozen.

2. Add 180µl of lysozyme lysis buffer (3.6.G.1). Incubate at 37^{o}C for 1 hour, vortexing occasionally.

3. Add 25µl of Proteinase K stock solution and 200 µl of buffer AL lysis buffer (provided by extraction kit manufacturer), then mix by vortexing and incubate at 70^{o}C for 30 minutes. Vortex occasionally. Tissues should be well lysed by the end of 30 minutes.

4. Incubate at 95^{o}C for another 10 minutes. Vortex occasionally

5. Add 210µl of ethanol, mix thoroughly on vortex

6. Place a spin column in a 2ml collection tube. Place sample mixture over the filter in the spin column, being careful not to moisten the rim of the column. Close the cap and centrifuge at 6000 x g for 1minute at room temperature.

7. Place the spin column in a clean 2 ml collection tube and discard the tube containing the filtrate.

8. Carefully open spin column and add 500µl Buffer AW1 (wash buffer provided by kit manufacturer). Centrifuge again as described above.

9. Repeat steps 7 and 8, using 500µl Buffer AW2. Centrifuge at full speed for 3 minutes to dry the membrane.

10. Place spin column in clean 1.5 ml Micro centrifuge tube and add 200µl of Buffer AE (elution buffer provided by kit manufacturer) for elution of DNA (TE buffer pH 8.0 or water can also be used). Incubate for 5 minutes at room temperature. Centrifuge at 6000 x g for 1 minute.

11. Repeat step 10 so that the total volume of DNA is 400µl. Discard spin column and store DNA solution at -20-70^{o}C until use.

12. Quantify the amount of DNA extracted with a spectrophotometer (Chapter 6).
    a. 25-50 mg of fish kidney tissue should produce between 100 and 300 ng DNA per µl using this procedure. A greater concentration of DNA should be diluted with elution buffer before performing PCR.
    b. 50 µl ovarian fluid produces a much lower amount of DNA per µl using this procedure. Dilute the template if DNA exceeds 300 ng/µl.

b. Initial Amplification of *R. salmoninarum* DNA

i. General QA/QC Considerations must be considered before performing PCR (See Chapter 6 for more specific QA/QC considerations for PCR).

ii. Procedures for initial round:

1. Using Worksheet 3.A3.A "DNA Samples", Record appropriate data for each sample to be tested by PCR.

2. Using Worksheet 3.A3.B "Initial Amplification..." record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed and the amount of MM needed per reaction (40 µl). Add 4 to the number of samples so that there is enough to run controls.

3. Under UV Cabinet, add PCR reagents **except for sample DNA** to the MM tube in the order listed on Worksheet 3.A3.B, adding water first and Taq last. Keep all reagents cold during mixing, and return them to freezer immediately after use. Do not expose enzymes, Primers, or dNTP's to UV light.

a. Water to make a 40 µl total volume per reaction

b. PCR Buffer mix (1X)

c. $MgCl_2$ (1.5 mM per reaction)

d. dNTP mix (0.2 mM per reaction)

e. Primers (20 pmole each per reaction)

i. Forward 5' - A GCT TCG CAA GGT GAA GGG – 3'

ii. Reverse 5' – GC AAC AGG TTT ATT TGC CGG G – 3'

f. TAQ polymerase (2 units per reaction)

4. Place 40 µl of MM into each 0.5ml PCR tube and close caps tightly. Move PCR tubes to sample loading area.

5. In sample loading area, load 10µl of each sample DNA to the appropriately labeled PCR tubes. Close caps tightly. Remove sample tubes from UV cabinet to thermocycler.

6. Load the sample tubes into the thermocycler wells.

7. Thermocycler should be programmed for 30-40 cycles of the following temperature regime, and recorded on Worksheet 3.A3.B:

Preheat sample to 94°C for two minutes.
Denaturing at 93°C for 30 seconds.
Annealing at 60°C for 30 seconds.
Extending at 72°C for 1 minute.
Post dwell at 4-16°C for holding samples after cycling is complete.

c. "Nested" PCR-secondary amplification of *R. salmoninarum* DNA - Materials, methods and general QA/QC considerations of this section and Chapter 6 also apply to the nested PCR process:

i. Using Worksheet 3.A3.C "Nested PCR" record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples and controls to be processed and the amount of MM needed for each reaction (49 µl).

ii. Add PCR reagents except the first round amplified DNA into the Master Mix (MM) tube. Return reagents to freezer

1. Primers:

a. Forward 5' – AT TCT TCC ACT TCA ACA GTA CAA GG – 3'

b. Reverse 5' – C ATT ATC GTT ACA CCC GAA ACC – 3'

iii. In PCR tubes (0.5 ml), pipette 49µl of MM. Close caps tightly. Remove tubes from UV cabinet to amplified DNA area

iv. Load 1µl of amplified sample DNA into the appropriate PCR tubes

v. Load PCR tubes into thermocycler wells

vi. Program thermocycler for 10-20 cycles of the following regime:

Preheat sample to 94°C for two minutes.
Denaturing at 93°C for 30 seconds.
Annealing at 60°C for 30 seconds.
Extending at 72°C for 1 minute.
Post dwell at 4-16°C for holding samples after cycling is complete.

Note: PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

d. Visualization of PCR Product by Electrophoresis – See Chapter 6 for general procedures.
    i. Visualization of amplified products resulting from PCR for detection of *R. salmoninarum* DNA is best accomplished after electrophoresis on a 1.5 or 2% agarose gel (6.3.C)

    ii. Using Worksheet 3.A3.C "Gel Preparation and Lane Designation", record location of each sample on the agarose gel at the time samples are loaded.

    iii. After electrophoresis, stain gel with ethidium bromide, and visualize on an UV transilluminator.

    iv. Carefully record locations of bands on positive control samples in relation to DNA ladder bands.

        1. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays (first round primer M21=383bp; nested primer M38=320 bp). **Bands occurring at these locations are confirmatory for *R. salmoninarum* and are reported as POSITIVE.**

        2. Note any unusual band occurrences. Negative controls should not have any bands. Suspicion of contamination indicates PCR should be re-run on samples from template DNA tube.

e. Document the electrophoresis results (6.3.G) - photograph all gels and attach the photo to Worksheet 3.A3.D. Attach to case history information**.**

3.6 Reagents, Media and Media Preparation

A. Growth Media – Most bacteriological culture medias are commercially available as pre-made or as bases, which can be easily prepared in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations. Recipes for those media which are not available in a commercial preparation are included below:

1. **Brain Heart Infusion Agar (BHIA)** (Difco 1998) - A basic agar for most bacterial cultures.

2. **Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB)** (Difco 1998) – A basic media for most bacterial cultures.

3. **Selective Kidney Disease Medium-2 (SKDM-2)** (Austin, et. al. 1983) - for selective isolation of *Renibacterium salmoninarum*

Peptone	10	gm
Yeast Extract	0.5	gm
L-Cysteine HCL	1	gm
Agar	15	gm
Distilled Water	874	ml

Adjust pH to 6.5 before adding agar. Autoclave for 15 minutes at 121^{o}C. Cool to ~ 50^{o}C and add:

Fetal Bovine Serum 100.0 mls

The following antibiotics are added to reduce overgrowth from other bacterial organisms):

4.0 mls Cyclohexamide (1.2 gms Cyclohexamide in 96 mls dH_2O)
1.0 ml D-Cycloserine (0.3 gms D-Cycloserine in 24 mls of dH_2O)
2.0 mls Polymyxin B-sulfate (0.3 gms Polymyxin B-sulfate in 24 mls of dH_2O)
1.0 mls Oxolinic Acid (0.06 gms Oxolinic Acid in 24 mls of 5% NaOH)

B Media to Identify Growth and Biochemical Characteristics - Most of these media are commercially available as pre-made or as bases, which can be easily prepared in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations. Unless otherwise specified, these media can also be prepared from basic laboratory ingredients according to recipes found in the following references: MacFaddin 1980, MacFaddin 2000, and Difco 1998.

1. **Motility Test Medium** - A semi-solid media used as a tube test to detect the ability of a microorganism to exhibit motility. Several types are commercially available, including MIO (motility, indole, ornithine) which allows for the detection of motility, and the reaction of two biochemical tests in the same tube.

2. **Trytic soy broth(TSB)** – a nutrient broth media used to determine motility of a microorganism with the hanging drop method.

3. **Triple sugar iron agar (TSI)** – A commercially prepared dehydrated media used to evaluate the utilization of glucose and two additional carbohydrates, as well as the production of hydrogen sulfide.

4. **Oxidation/Fermentation (OF) medium – A basal media** for Carbohydrate Utilization Tests, available in a commercially prepared dehydrated powder. The OF basal is prepared according to manufacturer's recommendations prior to the addition of individual carbohydrates as described below:

   To prepare final medium aseptically add 10 ml of a filter-sterilized (0.45 μm) 10% carbohydrate solution to autoclaved and cooled (50°C) media resulting in a 1% final concentration, with the exception of salicin, which should be made as a 5% solution resulting in a 0.5% final concentration(see below). Only one carbohydrate is added to the basal medium for each test to be run.

   10% Arabinose (1 gm Arabinose to 10 ml in dH_2O)
   10% Rhamnose (1 gm Rhamnose to 10 ml in dH_2O)
   10% Sucrose (1 gm Sucrose to 10 ml in dH_2O)
   10% Sorbitol (1 gm Sorbitol to 10 ml in dH_2O)*
   5% Salicin (0.5 gm Salicin to 10 ml in dH_2O)

   Mix flask thoroughly and aseptically dispense into sterile tubes. Store at 2-8°C. Final pH = 6.8 $\pm$ 0.2 at 25°C.

   *A sorbitol utilization slant media can also be prepared and utilized as described in Cipriano and Pyle, 1985.

5. **Nutrient Gelatin –** A dehydrated medium for determining the presence of Gelatinase.

6. **Tryptone Broth** – for use with the Indole test.

Tryptone	10 gm
Distilled Water	1000 ml

   Heat gently to dissolve. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Store at 2-8°C.

7. **Decarboxylase Medium Base** – A basal media for use in Lysine test.

   The basal media, without addition of lysine, serves as the control.

   To make L-Lysine media add 5 gm L-Lysine to 1 liter of prepare basal

decarboxylase media. Dispense into tubes. Autoclave at 15 pounds pressure for 15 minutes and allow tubes to cool. Final pH = 6.8 ± 0.2 at 25°C. Store at 2-8°C.

8. **Malonate Broth –** A media used for the malonate test.

9. **Bile Esculin Agar** – A media used for the esculin Test

C Media Preparation

1. **Plate Media**
   a. Prepare media in stainless steel beakers or clean glassware according to manufacturer instructions. Check pH and adjust if necessary. Media must be boiled for one minute to completely suspend agar. Use of a stir bar will facilitate mixing of agar.

   b. Cover beaker with foil, or pour into clean bottles being sure to leave lids loose. Sterilize according to manufacturer's instructions when given, or at 121°C for 15 minutes at 15 pounds pressure (consult with the manual provided by the autoclave manufacturer for adjustment of time when large volumes of media are being sterilized).

   c. Cool media to 50°C.

   d. Alternatively, media can be autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with media type, date and initials. When media is needed, boil, microwave or use a water bath to completely melt the agar. Cool to 50°C.

   e. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface. Mix any added ingredients into the media at this temperature.

   f. Label the bottom of each plate with medium type and date prepared.

   g. Remove bottle cap and pour plates or dispense with a sterile Cornwall pipette, lifting each petri dish lid as you go. Pour approximately 15 to 20 ml per 100 X 15mm petri dish. Replace lids as soon as the plate is poured.

   h. Invert plates when the media has cooled completely (~ 30-60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid.

   i. Allow plates to sit overnight at room temperature. Store plates upside down in the refrigerator in a tightly sealed plastic bag or plate storage tin.

   j. Follow manufacturer's recommendation for storage period of prepared media. Each batch should be labeled with date of preparation.

2. **Tube Media**
    a. Prepare media in stainless steel beakers or clean glassware according to manufacturer's instructions. If the medium contains agar, boil for one minute to completely suspend the agar. Use of a stir bar will facilitate mixing of agar.

    b. Media with indicators must be pH adjusted. This can be done when medium is at room temperature; otherwise compensation for temperature needs to be made.

    c. Arrange test tubes in racks. Disposable, autoclavable screw cap tubes can be used for all tube media.

    d. Use an automatic pipetter or Pipette-aid™ to dispense the medium. If using the Brewer or Cornwall pipette prime with deionized water, then pump the water out of the syringe prior to pipetting and discard the first few tubes of media that are dispensed. Dispense approximately 5 to 10 ml media in 16 X 125mm or 20 X 125mm tubes. Close caps loosely.

    e. Immediately after use, rinse the automatic pipetter in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.

    f. Loosely place screw caps on tubes. Do not tighten caps. It is necessary to allow pressure to release from tubes while heating in the autoclave.

    g. Follow manufacturer's recommendation for autoclave time and temperature.

    h. If making slants, put tubes in slant racks after autoclaving. Adjust the slant angle to achieve the desired slant angle and butt length (i.e. long butt and short slant for TSI or a standard slant over ¾ of the tube length for TSA or BHIA). Then tighten caps.

    i. Cool completely to room temperature in the slanted position.

    j. Label the tubes or the tube rack with type of medium and date made.

    k. Store at 2-8°C, following manufacturer's recommendation for period of long-term storage.

D Reagents-Most of these reagents are commercially available pre-made. These commercial products are entirely acceptable and should be stored according to the manufacturer's recommendations. The formulations provided below were obtained from the references cited.

1. **Gram Stain Reagents** - These stains can be ordered as a complete kit or can be prepared as follows:

a. Crystal violet

crystal violet (90% dye content)	20.0 g
ethanol (95%)	200 ml
ammonium oxalate	8.0 g
dH_20	800 ml

Mix first two ingredients and let sit overnight or until dye goes into solution. Add remaining ingredients and filter before use.

b. Gram's iodine

iodine crystals	1.0 g
potassium iodide	2.0 g
dH_20	300 ml

c. De-colorizer

acetone	40 ml
ethanol (95%)	60 ml

d. Safranin

safranin O	2.5 g
95% ethanol	100 ml
dH_20	900 ml

Filter safranin solution before use.

2. **FAT Mounting Fluid (pH 9.0):**

Glycerol	90.0 ml
DABCO*	2.5 g
PBS	10.0 ml

Suspend the DABCO in glycerol over low heat. Then add 1X PBS (see below). Adjust pH to 8.6 - 9.0 with 1N Hydrochloric Acid or 0.1N Sodium Hydroxide. The pH of the mounting media is important, as an acid pH will quench fluorescence. Check the pH frequently. Store at room temperature.
* Optional ingredient - DABCO is 1,4-diazabicyclo-(2,2,2)-octane. Its addition to mounting fluid can reduce quenching of fluorescence.

3. **Phosphate-buffered saline for FAT (PBS), pH 7.1**

a. 1× concentration (0.15 M NaCl, 0.01 M phosphate; makes 1 L)

NaCl	8.50 g
Na_2HPO_4 (anhydrous).....	1.07 g

$NaH_2PO_4 \cdot H_2O$ (monohydrate)	0.34 g
DH_2O	to 1 L

Adjust pH to 7.1 with 1N Hydrochloric Acid or 0.1N Sodium Hydroxide.

b. 5× concentration (makes 10 L of 1× PBS)

NaCl	85.00 g
Na_2HPO_4 (anhydrous).....	10.70 g
$NaH_2PO_4 \cdot H_2O$ (monohydrate)	3.45 g
DH_2O	to 2 L

Adjust pH to 7.1 with 1N Hydrochloric Acid or 0.1N Sodium Hydroxide.

4. **Kovac's Indole reagent**

Isoamyl alcohol	30 ml
p-Dimethyl aminobenzaldehyde	2 g
Hydrochloric Acid (HCl)	10ml

Dissolve the aldehyde in the alcohol. Slowly add the acid to the mixture. Store solution at 2-8°C in amber dropper bottle.

5. **Counter Stains**
   a. Rhodamine - Rehydrate the rhodamine to 1 mg/ml in distilled water. If the rhodamine does not completely dissolve, add a small drop of 0.1 M Sodium Hydroxide. Store at 2-8°C

   b. Evans Blue – Prepare 0.1% stock solution with distilled water and decontaminate with 0.45 µm membrane filtration. Store at room temperature. Prepare a 0.01% working dilution with sterile PBS (3.6.D.3). (Cvitanich, 1994)

   c. Eriochrome black T – prepare a solution at 1:60 (w/v) in PBS (3.6.D.3), and filter through Whatman #1 and Whatman # 42 filter papers before initial use (Elliott & McKibben, 1997)

6. **FITC Conjugated Rabbit Anti-X /Rhodamine Counter Stain**

Rhodamine stock	10.0 µl
FITC conjugated antibodies	10.0 µl
Phosphate buffered saline	480.0 µl

Store at 4°C.
Note: Evans Blue or Eriochrome black T can also be used as counterstain in FAT in a separate step during the staining process, but neither can be added directly to the conjugate solution.

E. Cytochrome Oxidase Spot Test - Individual test strips can be purchased from several suppliers (catalogue # 38-191, Remel, Tel. 800-255-6730).

F. Determination of Antiserum and Conjugate Working Dilutions for FAT

Commercially prepared anti-sera and conjugates are should be reconstituted according to the manufacturer's instructions. Aliquots of 0.5 ml can be frozen for later dilution into a working solution of the reagent. Reagents are more stable if frozen as a stock solution. The proper working dilution is the highest dilution that still maintains maximum brightness of the fluorochrome on positive control FAT preparations. Generally the manufacturer will recommend between 1:20 to 1:50 using a suitable buffer (PBS) as the diluent. However, in all cases each laboratory must establish the proper working dilution by starting with the manufacturer's recommendation and bracketing, or testing dilutions on either side of the recommended concentration. The following example shows how to determine the correct working dilution of FAT conjugate where the manufacturer recommends a working dilution of 1:40.

1. Using the stock solution, dilute the antiserum at 1:20, 1:30, 1:40, 1:50, 1:60 using PBS or another buffer as recommended by antibody manufacturer.

2. Rhodamine counter stain is added directly to the optimum antiserum working dilution at a 1:50 (alternatively, Evan's Blue may be used to counterstain FAT stained slides – do not add Evan's blue to the conjugate directly).

3. The FAT is performed on replicates of a known positive control, each replicate using a different dilution of the conjugated antiserum. In this way the working dilution can be determined as the endpoint of optimum fluorescence (the highest dilution that still provides a bright specific fluorescence with little or no background staining).

4. Prepared conjugate solutions should be filtered through 0.2 to 0.4 micron filter prior to use and storage. Store frozen in small aliquots. Do not thaw and re-freeze antibodies repeatedly.

G. PCR Reagents – the following are formulations for extraction reagents used specifically in the protocols described in 3.5.E.2.

1. **Lysozyme lysis buffer** – 100 mLs (this formulation is used for lysis of gram positive bacteria with Qiagen extraction kits)

Lysozyme	2 g
Tris HCl Stock	2 mLs
EDTA stock	2 mLs

Triton	1.2 mL

Bring these components to 100 mL with sterile distilled water (molecular grade)

2. **Tris HCl Stock solutions** – 100 mLs at 1M pH 8.0 (for use in lysozyme buffer)

Trizma base	5.7 g
Tris HCl	8.9 g
dH2O	85.4 mL

3. **EDTA Stock solution** – 100 mLs at 0.1 M (for use in lysozyme buffer)

EDTA	3.72 g

Bring to 100 mL with sterile distilled water (molecular grade).

3.7 Bacterial Identification Techniques

Unless otherwise specified, all of the materials and techniques described in Section 3.7 are described in detail in MacFaddin's (2000) and or the 11th Edition Difco Manual (1998). Each of the tests listed are provided with a set of control bacterial species available from ATCC, which will provide quality control for each biochemical test. It is not necessary, however, to set up control isolates for every test run in these protocols. It is strongly suggested that newly prepared batches of media and reagents be tested using the control bacterial isolates listed for each.

A. Gram Reaction - Gram staining detects a fundamental difference in the cell wall composition of bacteria.

1. **Gram Stain**: (Kits are available commercially, or formulas for reagents are listed in 3.6.A.)
    a. Prepare a bacterial smear from a pure culture
        i. Put a drop of saline, distilled water, PBS (3.6.D.3), or formalin saline (0.4% formalin, 0.85% NaCl) on a clean glass slide

        ii. Using a sterile loop or needle touch an isolated colony and mix in the water drop.

        iii. Mix until just slightly turbid (light inoculum is best, excess bacteria will not stain properly).

        iv. Let air dry and heat fix. Do not overheat; slide should not be too hot to touch.

        v. Allow to cool.

    b. Flood the slide with crystal violet (3.6.D.1.a), and allow to remain on the slide for 60 seconds

    c. Wash off the crystal violet with running tap water.

    d. Flood the slide with Gram's iodine (3.6.D.1.b), and allow to remain on the slide for 60 seconds.

    e. Wash off with running tap water.

    f. Decolorize with decolorizer solution (3.6.D.1.c) until the solvent flows colorless from the slide (approximate 5-10 seconds). Excessive decolorization should be avoided since it may result in a false gram-negative reading. Too little decolorization can result in a false positive result.

    g. Rinse immediately with running tap water.

h. Counter stain with Safranin (3.6.D.1.d) for 60 seconds.

i. Rinse with tap water and allow to air dry.

j. RESULTS:
   i. Gram negative: cells are decolorized by the alcohol-acetone solution and take on a pink to red color when counter stained with safranin.

   ii. Gram positive: cells retain the crystal violet and remain purple to dark blue.

k. Quality Control: Use a known bacterial culture as a control for each case history to assess correct staining and interpretation (cultures can be obtained from ATCC and maintained at 2-8°C for long term use)
   i. Positive: *Staphylococcus sp.* (ATCC – any isolate)

   ii. Negative: *Yersinia ruckeri*

   iii. Commercially prepared Gram stain control slides are available (Fisher Scientific, #08-801)

2. **3% Potassium Hydroxide -** Alternative test for Gram reaction
   a. Add a heavy inoculum of a pure culture of bacteria grown on a solid medium to a drop of 3% potassium hydroxide (KOH) solution (3 grams KOH per 100 mls distilled water) on a clean glass slide.

   b. Stir for about one minute, occasionally lifting the loop to look for thickening and "stringing" of the slurry.

   c. RESULTS:
      i. Gram-positive bacteria will not appear to change the viscosity of the KOH solution.

      ii. Gram negative bacteria will cause the KOH solution to become stringy or mucoid in appearance and consistency

   d. QUALITY CONTROL: Use a known bacterial culture as a control for each case history to assess correct staining and interpretation (cultures can be obtained from ATCC and maintained at 2-8°C for long term use)
      i. Gram Positive: *Staphylococcus sp.*

      ii. Gram Negative: *Yersinia ruckeri*

B. Cytochrome oxidase (3.6.E) – this test determines the presence of cytochrome oxidase enzymes. The use of an iron-containing metal inoculation loop can lead to a false-positive reaction. Use only plastic or platinum loops for this test.

1. Add an inoculum of a pure 18-24 hour old bacterial culture to the surface of the test strip impregnated with reagent.
2. Results:
    a. Positive: purple color within 5-10 seconds (reactions that occur after 10 seconds are negative).

    b. Negative: no purple color.

3. Quality Control:
    a. Positive: *Pseudomonas aerugenosa* (ATCC 10145)

    b. Negative: *Yersinia ruckeri*

    c. Observe expiration dates of reagent strips.

C. Motility – this test determines if a bacterial isolate is motile by means of flagella.

1. Hanging Drop Method
    a. Inoculate a tryptic soy broth (TSB (3.6.A.2)) with the isolate.

    b. Incubate at room temperature until growth is obtained, usually 24 hours.

    c. After incubation use a sterile loop or sterile dropper and place a drop on a clean cover slip.

    d. Place a tiny drop of distilled water in one corner of the same cover slip.

    e. Carefully invert the cover slip and place over the concave portion of a hanging drop slide.

    f. Observe for motility at 400X magnification on a compound microscope. Care should be taken to not interpret "drift" or "Brownian motion" as motility.

    g. Record results as motile or non-motile.

2. Semi-Solid Medium Method (3.6.B.1)
    a. Stab the semi-solid medium with a small amount of inoculum.

    b. Incubate overnight at room temperature.

    c. If the bacterial species is motile, the medium will become turbid with growth that radiates from the line of inoculum. If the bacterial species is non-motile, only the stab line will have visible bacterial growth.

d. Confirmation of results using the hanging drop method is recommended.

3. Quality Control:
   a. Positive: *Escherichia coli* (ATCC 25922)

   b. Negative: *Aeromonas salmonicida*

D. Biochemical Testing

1. Tube Method
   a. **Glucose Fermentation** - An OF basal medium (3.6.B.4) is used to test the fermentation of glucose by bacterial isolates.
      i. With a sterile needle inoculate two tubes of OF-glucose by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube. Screw the cap on loosely.
         1. Fermentation Test - One tube is over-layed with sterile mineral oil or paraffin. Sterile petroleum jelly (heated to melting) should be used for more accurate observation of gas production.

         2. Oxidation Test – The second tube is not overlayed.

      ii. Incubate at 20-24^{o}C and read after 18-24 hours.

      iii. Results:
         A = Acid (yellow)
         AG = Acid + Gas
         N = No change or Alkaline (green or blue-green)

	Fermentation Test Tube	Oxidation Test Tube
Fermentative	A or AG	A or AG
Oxidative	N	A or AG
Non-reactive	N	N

      iv. Quality Control:
         1. Fermentative: *Aeromonas* species
         2. Oxidative: *Pseudomonas fluorescens*

   b. **Triple Sugar Iron (TSI)** - TSI agar (3.6.B.3) contains the three sugars in varying concentrations: glucose (1X), which is a simple monosaccharide, and lactose and sucrose (10X each), both of which are disaccharides. It also contains the pH indicator phenol red. All organisms that utilize glucose will yield an initial acidic reaction throughout the tube (yellow - see below) regardless of whether they utilize sucrose or lactose. Reversion will not occur until all the glucose in the slant portion is completely utilized. At this point, the bacterium will utilize either one or both of the disaccharides, continuing the

production of acids in the media (the slant remains yellow). If, after all the glucose in the slant is used and the bacterium cannot utilize either lactose or sucrose, the bacterium is forced to revert to protein (peptone) present in the agar. In this case, nitrogenous bi-products are produced and the pH in the media rises until the pH indicator shows a reversion from yellow (acid) to red (alkaline). Blackened medium is caused by hydrogen sulfide production, which changes ferrous sulfate to ferrous sulfide. In addition, splitting of the medium or presence of bubbles in the butt of the tube can determine gas production.

i. With a sterile needle inoculate the TSI slant by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube. Screw the cap on loosely.

ii. Incubate at 20-24°C. Read after 18-24 hours.

iii. Results:
A = Acid
K = Alkaline
H_2S = Hydrogen sulfide produced
N =No change

Slant / Butt	Color	Interpretation
K / N or K / A	Red / Orange	Only Peptone Utilized
	Red/ Yellow	Only Glucose Fermented
A /A	Yellow /Yellow	Glucose plus Lactose and/or Sucrose Fermented
Gas	Splitting or Bubbles	Gas Production
H_2S	Black Butt	Hydrogen Sulfide Produced

iv. Quality Control:
1. K/A: *Shigella flexineri* (ATCC 12022)

2. A/AG: *Escherichia coli* (ATCC 25922)

c. **Gelatinase**– A test to determine bacterial production of gelatinases, enzymes that liquefy gelatin.

i. Inoculate by stabbing ½ to 1 inch deep into the Nutrient Gelatin media (3.6.B.5) with a heavy inoculum from an 18-24 hour pure culture.

ii. Incubate 18-24 hours at 20°C.

iii. Results:
1. Positive (+) - Media is liquefied. Weak results can be visualized by rapping the tube against the palm of the hand to dislodge droplets of liquid from the media. Any drops seen are considered positive.

2. Negative (-) - No liquefaction occurs in media.

iv. Quality Control:
    1. Positive: *Proteus vulgaris* (ATCC 8427)

    2. Negative: *Escherichia coli* (ATCC 25922)

v. Precautions:
    1. The liquid will generally appear turbid due to bacterial growth.

    2. Nutrient Gelatin softens at temperatures above 20°C. Keep refrigerated until ready to inoculate, and do not let tubes reach room temperature or warmer. This will make interpretation of results difficult. Tests, which are incubated at 35°C, should be refrigerated prior to recording results.

d. **Indole Test** - A test to determine bacterial ability to split indole from the tryptophan molecule. Certain bacteria are able to oxidize the amino acid, tryptophan, with tryptophanase enzymes to form three indolic metabolites - indole, skatole (methyl indole) and indoleacetate. Indole, pyruvic acid, ammonia, and energy are principle degradation products of tryptophan. Indole, when split from the tryptophan molecule, can be detected with the addition of Kovac's reagent. The reagent is not a dye or stain, but reacts with indole to produce an AZO dye.
    i. Inoculate tryptone broth (3.6.B.6) with a light inoculum from an 18-24 hour pure culture.

    ii. Incubate 24 to 48 hours at 20°C

    iii. At the end of 24 hours incubation –
        1. Aseptically remove 2 ml of media and place in an empty sterile test tube. Save extra tube for 48-hour incubation, if necessary.

        2. Add about 5 drops of Kovac's reagent (3.6.D.4) to one of the tubes and agitate tube.

        3. If a positive reaction is observed, the test is complete.

        4. If the 24 hour incubated sample is negative, incubate the remaining tube for an additional 24 hours, and test again for the presence of indole with Kovac's reagent.

    iv. Results:
        1. Positive (+) - within 1 to 2 minutes a cherry red ring will form at the surface of the media.

        2. Negative (-) - No color formation is observed at the surface, the

color remains that of the reagent – yellow.

3. Variable (V) - An orange color may develop. This indicates the presence of skatole, which may be a precursor of indole formation.

v. Quality Control:
   1. Positive: *Escherichia coli* (ATCC 25922)

   2. Negative: *Pseudomonas aerugenosa* (ATCC 27853)

vi. Precautions:
   1. Avoid inhaling fumes of Kovac's. Wear gloves to avoid skin contact.

   2. Tests for indole should be conducted after both 24 and 48 hours of incubation before a test can be declared negative. Split the broth culture prior to performing the 24-hour test. If negative, incubate the untested tube (without Kovac's) for another day and try again.

   3. Do not eliminate the 24-hour test, because some organisms may have produced indole by 24 hours, but have broken it down by 48 hours. DO BOTH!

   4. Kovac's reagent should be fresh. A color change from yellow to brown indicates aging and results in reduced sensitivity of the test.

   5. The procedure described here produces more reliable results than those obtained from MIO (motility-indole-ornithine) medium.

e. **Carbohydrate Utilization** (MacFaddin 1980)- the following carbohydrates are utilized to aid in bacterial species identification: Arabinose, Rhamnose, Mannitol, Salicin, Sorbitol, and Sucrose (saccharose). The procedures to be followed for each of these media are identical.
   i. Inoculate carbohydrate tube (3.6.B.4) with growth from an 18-24 hour pure culture.

   ii. Incubate with loosened cap 18-24 hours at 20^{o}C. A prolonged incubation of up to 4 days may be necessary for some negative results.

   iii. Results:

1. Positive (+) - Acid is produced from fermentation, which turns media yellow.

2. Negative (-) - No fermentation of carbohydrate, media remains green.

3. Aerogenic (G) - Gas bubbles are present within the media.

iv. Quality Control:

Carbohydrate	Positive Control Isolate	Negative Control Isolate
Arabinose	*Escherichia coli* (ATCC 25922)	*Yersinia ruckeri*
Sorbitol:	*Escherichia coli* (ATCC 25922)	*Y. ruckeri* Type I
Rhamnose	*Enterobacter aerogenes* (ATCC)	*Yersinia ruckeri*
Salicin	*Enterobacter aerogenes* (ATCC)	*Yersinia ruckeri*

v. Precautions:

1. Difficulty in interpreting test results may occur with slow growing bacteria. Prolonged incubation may be required.

2. Heavy bacterial growth throughout the media can offset the color of a negative (green) reaction, giving the appearance of a weakly positive (yellow) reaction. This is especially true with yellow-pigmented bacteria. These tubes should be retested if a true yellow color is not noted within several days.

f. **Decarboxylase Test (Lysine) -** A determination of bacterial enzymatic capability to decarboxylate an amino acid to form an amine with resultant alkalinity.

i. For each isolate to be tested, it is necessary to inoculate a Decarboxylase control tube and Lysine test tube (3.6.B.7). Use light inoculum from 18-24 hour pure culture.

ii. Add 1-2 ml oil overlay to each tube.

iii. Incubate 24 hours at 20^{o}C. A prolonged incubation of up to 4 days may be necessary.

iv. Results:

Test Result	Lysine Tube	Control Tube
Positive	Turbid to faded purple (glucose fermented, decarboxylase produced)	Yellow (glucose fermented)
Negative	Yellow (glucose fermented)	Yellow
Negative	Purple (glucose not fermented, decarboxylase not produced	Purple (glucose not fermented)

v. Quality Control:
   1. Positive: *Yersinia ruckeri*

   2. Negative: *Enterobacter cloacae* (ATCC 13047)

vi. Precautions:
   1. At the end of incubation, the Lysine tube might show a layer of purple over yellow. Gently shake the tube before interpreting the result.

   2. An indistinct yellow-purple color may be difficult to interpret. Use the control tube for comparison. Any trace of purple color after a 24-hour incubation in the amino acid tube denotes a positive result.

   3. Do not interpret tests prior to 18-24 hours. During the first 12 hours, only glucose is fermented which produces a yellow color. Decarboxylase enzymes do not form until the acidic environment is established by the fermentation of glucose.

g. **Malonate Test -** A method to establish if a bacterial isolate is able to utilize sodium malonate as its only source of carbon.
   i. Inoculate malonate media (3.6.B.8) with a light inoculum from an 18 - 24 hour pure culture.

   ii. Incubate 24 to 48 hours at 20^{o}C.

   iii. Results:
      1. Positive (+) - light blue to deep blue color throughout the media.

      2. Negative (-) - color remains the same as un-inoculated tube - green.

iv. Quality Control:
    1. Positive: *Enterobacter aerogenes* (ATCC 13048)

    2. Negative: *Yersinia ruckeri*

v. Precautions: The test tube must be incubated for at least 48 hours before it may be called negative. Since some bacteria produce only slight alkalinity, it is useful to compare the test to an un-inoculated tube. Any trace of blue color denotes a positive reaction.

h. **Esculin Test** - To determine the ability of an organism to hydrolyze the glycoside esculin (aesculin) to esculetin (aesculetin) and glucose in the presence of bile (10 to 40%).
    i. Inoculate the surface of the bile esculin slant (3.6.B.9) with inoculum from an 18 to 24 hr. old pure culture

    ii. Incubate 20° C for 24 to 48 hrs.

    iii. Results:
        1. Positive (+): presence of a black to dark brown color on the slant.

        2. Negative (-): No blackening of the medium.

    iv. Quality Control:
        1. Positive: *Enterobacter aerogenes* (ATCC 13048)

        2. Negative: *Yersinia ruckeri*

    v. Precautions: False positives may occur with hydrogen sulfide producing organisms, such as S*hewanella putrefaciens.* Neither of the target organisms for these protocols will, however, produce hydrogen sulfide.

2. **Commercial Identification Systems** – Several commercial test strips or kits are available for biochemical testing of bacteria. Bear in mind that these kits are designed for human and/or animal testing and the manufacturer's recommended incubation temperature is 37°C. The decreased incubation temperature (22°C- room temperature) required for most fish pathogens results in slightly different reactions and longer incubation periods. Therefore, test results may not follow the manufacturer's identification profiles exactly. Therefore, it may be necessary to refer to the Charts in 3.A2 for identification when API strips are employed.
    a. **API 20E™** – The API 20E™ system is a standardized, miniaturized version of conventional procedures for the identification of *Enterobacteriaceae* and other Gram-negative bacteria. It is a microtube system designed for the performance

of 23 standard biochemical tests from isolated colonies on plating medium. Refer to the instructions enclosed with each kit for more detailed information. The API system™ is available from bioMérieux (1-800-638-4835, catalog #20-109/20-179). Reference charts of API profiles for *A. salmonicida* and *Y. ruckeri* are listed in 3.A2.

b. **Biolog** – MicroLog™ is a microbial identification system able to identify and characterize a wide variety of organisms based on carbon source utilization. The system has identification databases that contain over 1400 different species/genera of aerobic and anaerobic bacteria and yeasts. The identification databases include a wide variety of organism including animal, plant, and water pathogens. The system also allows the user the capability to build customized organism databases. Products are available directly from Biolog, 3938 Trust Way, Hayward, CA. 94545 (1-510-785-2564 or website www.biolog.com).

E. Fluorescent Antibody Test (FAT) - The Fluorescent Antibody Test (FAT) is one serological method for corroboration testing of bacterial isolates. FAT can be performed either with a direct antibody staining (DFAT) or indirect (IFAT) technique, depending upon the availability of pathogen-specific FITC-conjugated or unconjugated antibody preparations from the manufacturers listed below (3.7.E.6). There are three basic steps for FAT: preparing and fixing bacterial samples; staining the slides with antibody reagents; reading and interpreting the slides.

1. Preparing the slides
    a. Pure Bacterial Cultures (confirmatory testing of pure isolates of *A. salmonicida*, *Y. ruckeri,* or *E. ictaluri*).
        i. Pure isolates of bacteria are diluted in sterile PBS (3.6.D.3) and applied to 2 replicate wells of an FAT slide.

        ii. Allow air-drying completely, or heat fix.

    b. Kidney (for presumptive detection of *R. salmoninarum*) – prepare kidney smear on a non-coated or acetone-cleaned glass slide
        i. Homogenize a piece of posterior kidney tissue and create a thin smear on the surface of a slide.

        ii. Allow to air dry completely, or heat fix.

    c. Ovarian Fluid pellet smear (DFAT-for presumptive detection of *R. salmoninarum*) – ovarian fluid is collected (2.2.E.2.f).
        i. After pooled ovarian fluid samples are processed and appropriate amount of supernatant removed for virology assays (4.4.C), the pellet is re-suspended in the remaining supernatant by thorough vortexing or repeat pipetting.

ii. Transfer two 1.5 mL aliquots from each pool of ovarian fluid (or 0.5 mL per fish) to sterile, labeled 1.5 mL micro-centrifuge tubes (see **Note**). Freeze the remainder of the sample at -20^{o}C for PCR confirmation.

iii. Centrifuge the 1.5 mL aliquots at 10,000 X g for 15 minutes.

Note: Elliot and McKibben (1997) document the importance of using a minimum of 10,000 X g for sufficient sedimentation of *R. salmoninarum* from 0.5 mL ovarian fluid collected from individual fish. Enough ovarian fluid must be transferred from each pool to represent 0.5 mL for each fish represented in the pool (i.e.2.5 mLs from a 5-fish-pool). Most 15 mL centrifuge tubes used for collection and processing of ovarian fluid samples cannot be centrifuged at a relative centrifugal force (rcf) as high as 10,000 X g. It is, therefore, necessary to aliquot the appropriate amount of fluid into microcentrifuge tubes suitable for the required rcf.

iv. The pellet is carefully and a thin smear prepared on a glass slide. Pellets originating from the same ovarian fluid sample tube may be combined on one slide.

v. Allow to air dry completely, or heat fix.

d. After the tissue is completely air dried or heat fixed, slides are fixed in acetone for 5 minutes. Other fixing solutions are suitable as long as a component of the solution contains a lipid dissolving reagent such as acetone or xylene, which helps remove lipids and improve the overall fluorescence quality and intensity.

2. **Direct FAT (DFAT) Staining** (Thoesen, 1994)- Once slides are prepared, the staining procedure is the same regardless of the sample type (bacterial isolate or tissue).
   a. Positive and Negative Controls: Slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for FAT staining. Positive controls are always used in confirmation testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and non-specific fluorescence.

   b. Place slides in dark, humidified chamber, and place one drop of specific FITC conjugate (3.6.D.6) on each sample slide and control slides.

   c. Incubate for 30-60 minutes at room temperature, according to manufacturer's recommendation.

d. Using a squirt bottle or transfer pipette, GENTLY rinse the slides with PBS (3.6.D.3).

e. If Rhodamine counterstain has not been incorporated in the FAT stain, apply the counterstain of choice (3.6.D.5) at this point for 1-2 minutes.

f. Final rinse/soak in PBS for 5-10 minutes. Air-dry completely.

g. Add a small drop of FA Mounting Fluid, pH 9 (3.6.D.2) to each slide, being careful not to touch the dropper to the slide to prevent the possibility of cross contamination.

h. Place a 24 x 50 mm cover slip over the slide using care not to trap air bubbles.

i. Spread the mounting fluid by gently pressing the cover slip with the blunt end of a pen or lab marker.

j. Add one drop of immersion oil to the cover slip and examine at 1000X using the epifluorescent filter.

3. **Indirect FAT (IFAT) Staining** (Thoesen, 1994) – IFAT is a double layered antibody technique, where the first layer consists of unconjugated purified, immunoglobulin (IgG) or antibody prepared in one animal species (e.g. rabbit) against the target antigen. The second antibody applied is a FITC-conjugated antibody prepared in a second animal species (e.g. goat), and specific for IgG of the first animal species (e.g. goat anti-rabbit IgG). Once slides are prepared, the staining procedure is the same regardless of the sample type (bacterial isolate or tissue).
    a. Positive and Negative Controls: Slides containing both a non-cross-reacting bacterial species and a known positive control can be prepared in quantity and stored refrigerated for use as controls for FAT staining. Positive controls are always used in corroboration testing to correctly identify morphology and fluorescence of the bacteria in question. The negative control is important in determining overall staining technique, background debris, and non-specific fluorescence.

    b. Place slides in dark, humidified chamber, and place one drop of unconjugated, pathogen specific antibody on each sample slide and control slides.

    c. Incubate at room temperature for 30-60 minutes, according to manufacturer's recommendations.

    d. Gently rinse slides with PBS (3.6.D.3).

e. Place a drop of FITC-conjugated second antibody on slides and incubate at room temperature in dark chamber according to manufacturer's recommendation.

f. Rinse briefly with PBS. If rhodamine counterstain as not been incorporated into the FAT stain, apply the counterstain of choice (3.6.D.5) at this point for 1-2 minutes.

g. Rinse and wash in PBS for 5-10 minutes.

h. Air dry completely and apply a small drop of FA mounting fluid, pH 9 (3.6.D.2). Apply a cover slip to mounting fluid.

i. Add one drop of immersion oil to the coverslip and examine at 1000X using the epifluorescent filter.

4. Reading Results:
    a. Slides are read at 1000x on a compound fluorescence microscope (refer to the microscope manufacturer for correct wavelength and filters required for FITC epifluoresence microscopy). The positive and negative control slides are read first. This has two purposes: (1) quality assurance for the staining process (positive control should have myriad numbers of fluorescing bacteria, the negative control should have no fluorescence); and (2) to familiarize the reader with the correct bacterial size, shape, and magnitude of the fluorescent halo of bacteria in the positive control. The reader can refer back to the positive control as a reference, if needed, to confirm suspect bacteria in the sample wells. Positive fluorescence appears "apple green" in color.
        i. Pure Culture Bacterial Confirmatory Testing: Positive bacterial isolates will fluoresce strongly and have the same morphology and size as the positive control. Negative isolates will not fluoresce.

        ii. *R. salmoninarum* Screening: Examine at least 50 fields. Positive bacterial cells are 1.0 X 0.5 microns, which fluoresce. Negative smears will not fluoresce.

5. Hints for Good Results:
    a. Use FITC conjugates at optimum working dilution. Follow manufacturer's recommendation to test for optimum working concentration (3.6.F).

    b. Filter all conjugated antibody reagents (.45um filter) prior to use to reduce background debris that fluoresce nonspecifically and cause difficulty in reading and interpreting the slides.

    c. Prepare thin smears; thick smears will not fix properly and are more easily washed off during the staining process, and thick slides require frequent focusing to observe multiple focal planes.

d. Evenly distribute the kidney material in PBS (3.6.D.3), or use a very light inoculum of pure bacterial culture for each well (excess bacteria will stain poorly).

e. Heat-fix slides prior to fixing in acetone. If there is not possible to heat-fix the slides they can be air-dried and sent to the lab without fixation. Fixed slides should be stored refrigerated until stained.

f. Control slides should be rinsed in separate Coplin jars to avoid any potential for cross contamination during the staining process. Fixing solutions should be completely changed at a minimum between separate inspection cases, and/or when positive results occur.

g. Use anhydrous acetone to fix slides; the acetone reduces the lipid content of the preparation (de-fatting) increasing the overall fluorescence quality and intensity.

6. Commercial Sources for Antibodies:
   a. DiagXotics, Inc.
      27 Cannon Rd., Wilton, CT 06897 USA
      Phone: 800-676-2927, 203-762-0279
      Web Site: http://www.diagxotics.com
      Antibodies Available: Monoclonal antibodies available in FITC-conjugated and other preparations for the following bacterial pathogens; *Renibacterium salmoninarum, Aeromonas salmonicida, Edwardsiella ictaluri.*

   b. Kirkegaard and Perry Laboratories, Inc.
      2 Cessna Court, Gaithersburg, MD 20879-4145 USA
      PHONE: 800-638-3167, 301-948-7755
      Web Site: http://www.kpl.com
      Antibodies available: Polyclonal antibodies available in FITC-conjugated and other preparations for *Renibacterium salmoninarum* only. KPL also provides positive control material for FAT.

   c. Microtek International, LTD (Bayotek)
      6761 Kirkpatrick Crescent, Saanichton, B.C., CA
      Phone: 250-652-4482
      Web Site: http://www.microtek-intl..com
      Antibodies available: Polyclonal antibodies available for Indirect FAT for he following bacterial pathogens; ; *Renibacterium salmoninarum, Aeromonas salmonicida,Yersinia ruckeri* serotypes 1 and 2

3.8 Glossary

Acute – a disease having rapid onset, severe symptoms and a relatively short course – not chronic.

Anneal – the attachment of oligonucleotide primers to a specific site on a single stranded DNA segment.

Aseptic – the nature of preventing contamination of foreign microorganisms.

Basal media – a media formulation to which additional components may be added for a particular test.

Carbohydrate – a particular sugar used in a test.

Chronic – describes the course of a disease which is long and drawn out – not acute.

Commercially prepared – a component or test which is available from a commercial source for purchase.

Confirmatory identification – identification of a pathogen through the completion of both initial screening techniques, and another confirmatory test as described in this document, the results of which concur with each other for positive identification of a particular organism.

Counterstain – a stain used in FAT to achieve a dark background color of tissues and materials stained, aiding in the observation of fluorescing bacterial cells.

Decolorize – the application of a solution to stained material to remove excessive stain.

Denature – the enzymatic/temperature dependent activity which converts double stranded DNA to single stranded DNA.

DNA extraction – the process of obtaining pure, double stranded DNA from sample tissues and materials.

Elution – the washing of DNA from a spin column filter membrane.

Extension – the synthesis of a new, copied segment of DNA following denaturing and annealing processes involved with PCR.

Fermentation – bacterial utilization of a compound in the absence of oxygen.

Filtrate – the resultant liquid obtained from filtration

FITC – fluorescein isothiocyanate, a reagent which is used as an antibody label for the fluorescent antibody test.

FITC-conjugated (antibody) – describes the existence of a fluorescent label on an antibody used for the fluorescent antibody test.

Lysate – the product solution of tissue lysis.

Nested-PCR – a second PCR is performed, targeting a sub-segment of DNA produced in the first round PCR specific for a particular organism.

Oxidation – bacterial utilization of a compound in the presence of oxygen.

Pellet – the product of sedimentation of solid materials from a liquid resulting from centrifugation.

Phenotypic characterization – the detectable expression of a bacterial isolate to environmental conditions, biochemical testing and morphological observations recorded for the purpose of differentiation and determination of the species of bacteria.

Polymerase chain reaction (PCR) – a rapid procedure for in vitro enzymatic amplification of a specific segment of DNA.

Positive control isolates – bacterial isolates may be obtained from the American Type Culture Collection (ATCC: http://www.atcc.org).

Post-dwell – the period of time after cycles are completed in PCR.

Presumptive identifications – identification of a pathogen through the completion of initial screening tests as described in this document, where no other test has been performed to confirm the positive identification of the organism.

Primary Culture – bacterial cultures achieved from media inoculated directly from fish tissues.

Primer – oligonucleotides that, in the presence of DNA and excess dNTP's, hybridize specifically to a target sequence and "prime" new DNA synthesis.

Pure Bacterial Culture – a culture of bacteria originating from an isolated colony.

Quality Control – Taking steps to assure that testing and results are accurate and reliable.

Selective media – a medium containing ingredients which may either exclude growth of some microorganisms, select for growth of a particular species of microorganism, or both.

Serological methods – detection methods which employ the use of an antibody against the target organism.

Spin column – a small filter unit provided by many commercial DNA extraction kits used to

bind, wash and elute DNA from tissue lysates.

Stock suspension – suspension of a compound which must be further diluted before direct application in a particular test.

Sub-acute – a course of disease which exhibits some acute and some chronic symptoms.

Subculture – the transfer of an established bacterial culture to an uninoculated medium.

Supernatant – the surface fluid resulting from centrifugation of a liquid.

Working suspension (dilution) – suspension of a compound at the correct dilution for direct application in a particular test.

Yellow-pigmented bacteria – bacterial isolates which exhibit a yellow pigmentation on colony formations (e.g. *Flavobacterium* species).

3.9 References

Austin, B. D. Bucke, S. Feist and J. Rayment (1985) A false positive reaction in the indirect fluorescent antibody test for *Renibacterium salmoninarum* with a 'coryneform' organism. Bull Eur Assoc Fish Pathol 5:8-9

Austin, B. and D.A. Austin (1987). Bacterial Fish Pathogens, disease in farmed and wild fish. Ellis Horword LTD, West Sussex, England

Austin, B., T.M. Embley, and M. Goodfellow (1983) Selective isolation of *Renibacterium salmoninarum*. FEMS Microbiol. Letters 17:111-114

Brown, L.L., T.P.T. Evelyn, G.K. Iwama, W.S. Nelson, R.P. Levine (1995) Bacterial species other than *Renibacterium salmoninarum* cross-react with antisera against *R. salmoninarum* but are negative for the p57 gene of *R. salmoninarum* as detected by the polymerase chain reaction (PCR). Dis Aquat Org 21:227-231

Bullock, G.L., B.R. Griffin, and H.M. Stuckey (1980) Detection of *Corynebacterium salmonis* by direct fluorescent antibody test. Can J Fish Aquat Sci 37:719-721

Bullock, G.L., R.C. Cipriano, and S.F. Snieszko (1983) Furunculosis and other diseases caused by *Aeromonas salmonicida*. Fish Disease Leaflet 66, USDOI, Fish & Wildlife Service, Div. Fish Res., Wash. D.C. 20240

Bullock, G.L. (1984) Enteric redmouth disease of salmonids. Fish Disease Leaflet 67, USDOI, Fish & Wildlife Service, Div. Fish Res., Wash. D.C. 20240

Bullock, G.L. and R.L. Herman (1985) *Edwardsiella* infections of fishes. Fish Disease Leaflet 71, USDOI, Fish & Wildlife Service, Div. Fish Res., Wash. D.C. 20240

Bullock, G.L. and R.L. Herman (1988) Bacterial kidney disease of salmonid fishes caused by *Renibacterium salmoninarum.* Fish Disease Leaflet 78, USDOI, Fish & Wildlife Service, Div. Fish Res., Wash. D.C. 20240

Cipriano, R.C., J.B. Pyle (1985) Development of a culture medium for determination of Sorbitol utilization among strains of *Yersinia ruckeri*. Microbios Letters 28:79-82

Cipriano, R.C., C.E. Starliper, and J.H. Schachte (1985) Comparative sensitivities of diagnostic procedures used to detect bacterial kidney disease in salmonid fishes. J Wildl Dis, 21(2):144-148

Chapman, P.F., R.C. Cipriano, and J.D. Teska (1991) Isolation and phenotypic characterization of an oxidase-negative *Aeromonas salmonicida* causing furunculosis in coho salmon (*Onchorhyncus kisutch*). J. Wildl. Dis. 27:61-67

Chase, D.M. and R.J. Pascho (1998) Development of a nested polymerase reaction for

amplification of a sequence of the p57 gene of *Renibacterium salmoninarum* that provides a highly sensitive method for detection of the bacterium in salmonid kidney. Dis Aquat Org 34:223-229

Cvitanch, J.D. (1994) Improvements in the direct fluorescent antibody technique for the detection, identification, and quantification of Renibacterium salmoninarum in salmonid kidney smears. J. Aquat. Anim. Health 6(1): 1-12

Difco Manual, 11th Ed (1998) Difco Laboratories, Division of Becton Dickinson & Co., Sparks, Md. (This Manual is available on the internet at the following website: http://www.bd.com/industrial/difco/DifcoManual.pdf)

Elliott, D.G., and Barila, T.Y. (1987) Membrane filtration-fluorescent antibody staining procedure for detecting and quantifying *Renibacterium salmoninarum* in coelomic fluid of chinook salmon (*Oncorhynchus tshawytscha*). Can. J. Fish Aquat. Sci. 44:206-210.

Elliott, D.G., and McKibben, C.L. (1997) Comparison of two fluorescent antibody techniques (FATs) for detection and quantification of *Renibacterium salmoninarum* in coelomic fluid of spawning chinook salmon *Oncorhynchus tshawytscha*. Dis. Aquat. Org. 30:37-43.

Hawke, J.P., et al (1981) *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish. Int. J. Syst. Bacteriol. 31: 396-400

MacFaddin, Jean F. (2000) Biochemical Tests for Identification of Medical Bacteria, Third Ed. Lippincott Williams & Wilkins, 227 East Washington Square, Philadelphia, PA 19106

Pascho, R.J., Chase, D., and McKibben, C.L. (1998) Comparison of the membrane-filtration fluorescent antibody test, the enzyme-linked immunosorbent assay, and the polymerase chain reaction to detect *Renibacterium salmoninarum* in salmonid ovarian fluid. J. Vet. Diagn. Invest. 10:60-66.

Pascho, R.J., Elliott, D.G., Mallett, R.W., and Mulcahy, D. (1987) Comparison of five techniques for the detection of *Renibacterium salmoninarum* in coho salmon. Trans. Am. Fish. Soc. 116(6):882-890.

Paterson, W.D. et al. (1980) Relationships between selected strains of typical and atypical *Aeromonas salmonicida, A. hydrophila*, and *Haemophilus piscium*. Can J. Microbio. 26:588-598

Plumb, J.A. and S. Vinitnantharat (1989) Biochemical, biophysical and serological homogeneity of *Edwardsiella ictaluri.* J. Aquat. Anim. Health 151-56

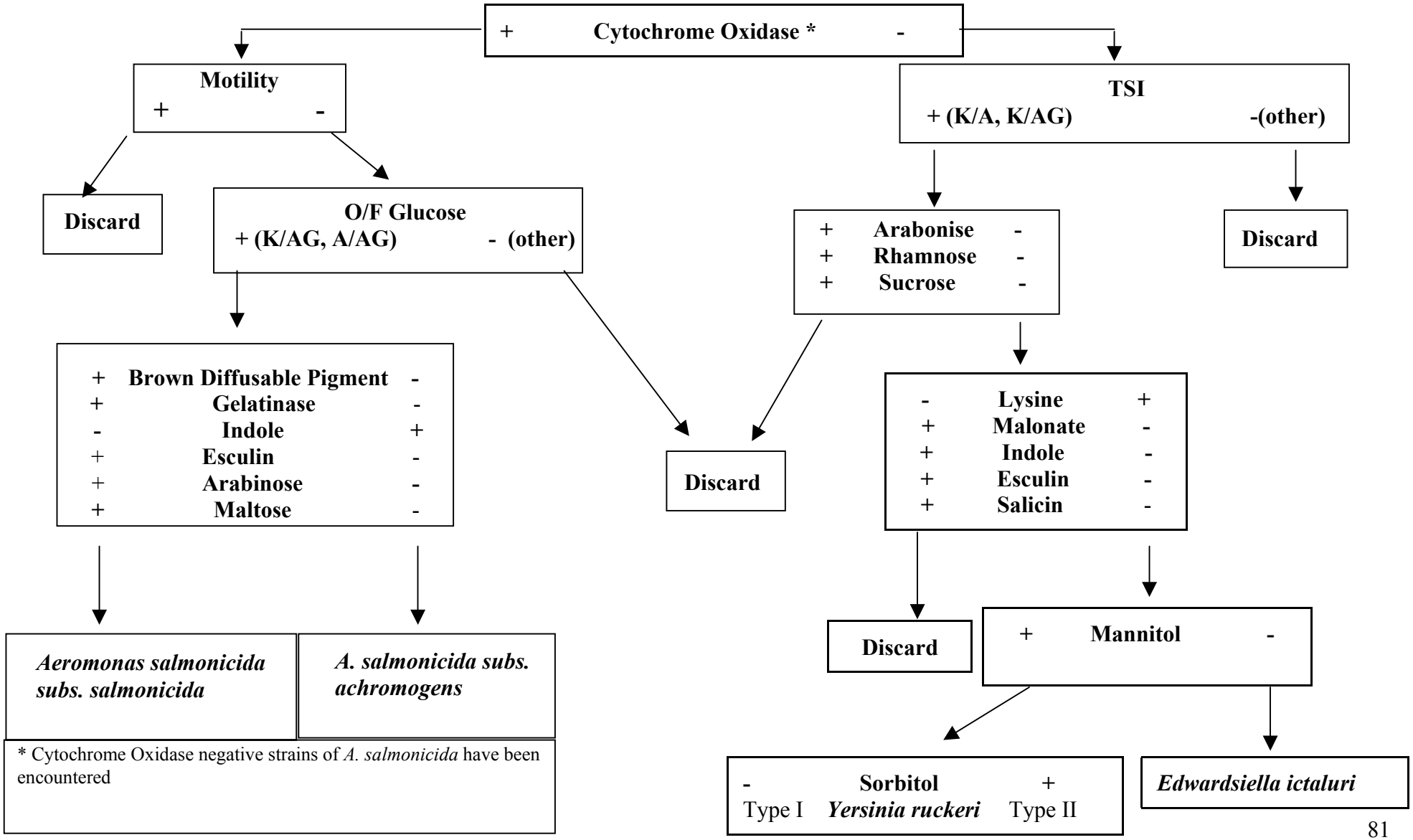
Plumb, J.A. and C. Shoemaker (1995) Effects of temperature and salt concentration on latent *Edwardsiella ictaluri* infections in channel catfish. Dis. Aquat. Org. 21:171-175

Sanders, J.E. and J.L. Fryer (1980) *Renibacterium salmoninarum*, the causative agent of

bacterial kidney disease in salmonid fishes. Int. J. Syst. Bacteriol. 30: 496-502

Thoesen, J.C. Editor (1994) Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. 4th ed., Version 1, Fish Health Section, American Fisheries Society.

3.A1 - Laboratory Reference Flow Chart for identification of Gram Negative Bacterial Pathogens which grow on TSA or BHIA and are targeted for detection to complete Fish Health Inspection requirements.



3.A2 - Profiles Obtained with API-20E for Known Fish Pathogens.

The following table represents API20E profiles for ***Yersinia ruckeri*** when cultures were tested at 22°C rather than the manufacturer's recommended incubation temperature of 35-37°C. All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT. Based on the profile submitted to API, bacterial identification is given in order of probability, then remarks as to the likelihood of the profile are provided when profiles are poorly matched to the manufacturer's database (National Wild Fish Health Survey Lab Procedures Manual, USFWS).

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #1- Type II (11.29) 2. Nisqually Fall chinook (3/88)	**5307500**	1.) *Serratia marcescens* 2.) *Serratia liquefaciens* 3.) *Hafnia alvei*
1. Eastern Fishery Disease Laboratory (EFDL) Positive Control #2- Type II (11.29) 2. Fall Chinook, Suquamish R, WA (3/88) 3. Spring Chinook, Skookum Creek, WA (2/88)	**5107500**	(Same ID as 5307500 above) 1.) *Serratia marcescens* 2.) *Serratia liquefaciens* 3.) *Hafnia alvei*
Unknown source – Isolate confirmed by biochemical and serological testing.	**5144100**	1.) *Escherichia coli* 2.) *Yersinia ruckeri*
Eastern Fishery Disease Laboratory (EFDL) Positive Control - Type I (11.4)	**5107100**	"Unacceptable profile"
Coho, Quinault River, WA	**5106100**	"Questionable ID"
Late Fall Chinook, Battle Creek, CA (11/94)	**5105100**	1.) *Hafnia alvei* "Acceptable ID"
Notes from ERM archived files – previous testing	**5104500**	"Questionable ID"

3.A2 (continued)

Bacterial Isolate ID	API PROFILE*	API Manual or Computer Identification
1. Hagerman – Type I (11.4)	**5104100**	1.) *Yersinia ruckeri* "Very good ID"
Coho, Quilcene R., WA (11/88)	**5104000**	1.) *Yersinia ruckeri* "Very good ID"
Unknown source	**5100100**	1.) *Yersinia ruckeri* "Excellent ID"
Unknown source	**4105100**	1.) *Hafnia alvei*
Unknown source	**4104100**	1) *Yersinia ruckeri* 2) *Salmonella gallinarum*
Unknown source	**4104000**	1.) *Yersinia ruckeri* "Acceptable ID"
Unknown source	**0104100**	1.) *Yersinia ruckeri* "Acceptable ID"

* *Yersinia ruckeri* generally fails to produce a positive citrate reaction when incubated at room temperature (22-25°C). Refer to the API Manual for specific biochemical tests and interpretation of API20E™ profiles. Also see references listed on page 5-27.

3.A2 (continued)

The following represents API20E profiles for ***Aeromonas salmonicida*** isolates following manufacturer's instructions but incubating test strips at room temperature (22°C). All isolates listed tested appropriately with the standard biochemical media and confirmed serologically by FAT.

Bacterial Isolate ID (collection date)	**API PROFILE***	**Computer/Manual ID**
spp not identified, Makah NFH, WA (8/88)	**0006104**	*Pseudomonas pseudomaleae* "Acceptable ID"
Winter Steelhead, Makah NFH, WA (1/89) Chum, Makah NFH , WA (12/89)	0006104	Same as above
Winter Steelhead, Quinault NFH, WA (1/89)	0006104	Same as above
Spring Chinook, Entiat NFH, WA (8/89)	0006104	Same as above
Spring Chinook, Quilcene NFH, WA (3/91)	**2006104**	*Aeromonas salmonicida*
Profiles given in **API MANUAL** for *Aeromonas salmonicida*	**6006104** **6006504** **4006104** **2006104**	*Aeromonas salmonicida* "Good to Excellent ID"

**Aeromonas salmonicida* generally fails to produce positive relations for ONPG, ADH, and LDC when incubated at room temperature (22-25° C).

Profiles provided in the API Manual are based on positive reactions for some or all of these first 3 biochemical tests, therefore the first digit of the "acceptable" profiles for *A.salmonicida* include the values 2,4, or 6. More often, a zero value is obtained after 24-48 hours incubation at room temperature. Longer incubation periods are required for these tests.

References:

Romalde, J.L., and A.E. Toranzo. 1991. Evaluation of the API-20E system for the routine identification of the enteric redmouth disease. Bull. Eur. Ass. Pathol. 11(4), 147.

Kent, M.L. 1982. Characteristics and identification of Pastuerella and Vibrio species pathogenic to fishes using API-20E (Analytab Products) multitube test strips. Can. J. Fish. Aquat. Sci.,

39. 1725-1729.

Toranzo, A.E., Y.Santos, T.P. Nieto and J.L. Barja. 1986. Evaluation of different assay systems for identification of environmental Aeromonas strains. Appl. Environ. Micorbiol., 51:652-656.

3.A3 PCR Worksheets

A. Worksheet A - PCR Sample Data/Log Sheet

PCR Sample Data/Log Sheet

Case Number_____ **Sample Site**_____ **Date**_____
Species_____
Tissue type_____

Tissue Sample ID	Extraction Method	PCR Result	Notes
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			
12.			
13.			
14.			
15.			
16.			
17.			
18.			
19.			
20.			

B. Worksheet B - Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum*

WORKSHEET 3.A3.B**: Initial Amplification of *R.salmoninarum* DNA by PCR**.

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		Add to total 40µL		30.1	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5mM	50mM	1.5	
dNTP's		0.2mM	10mM	1	
(+)Primer		20pMole	20pMole/µl	1	
(-)Primer		20pMole	20pMole/µl	1	
TAQ		2 units/Rx	5U/µl	0.4	
DNA$^{\pm}$		-	-	10 µl	-

*Add water to Master Mix first, TAQ last.

**Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations.

$^{\pm}$Do not add DNA template until Master Mix reaction tubes have been removed from the reagent Mixing (MM) area.

Primer Sets for *R. salmoninarum* 1st Round Amplification

P3 (round 1 forward)	5'-A GCT TCG CAA GGT GAA GGG-3'
M21 (round 1 reverse)	5'-GC AAC AGG TTT ATT TGC CGG G-3'

C. **Worksheet C - Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Renibacterium salmoninarum***

WORKSHEET 3.A3.C: Nested Amplification of *R.salmoninarum* DNA by PCR.

Case Number_____ Date_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration**	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		Add to total 49µL		39.1	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5mM	50mM	1.5	
dNTP's		0.4mM	10mM	1	
(+)Primer		20pMole	20pMole/µl	1	
(-)Primer		20pMole	20pMole/µl	1	
TAQ		2 units/Rx	5U/µl	0.4	
DNA$^{\pm}$ (round 1)		-	-	1 µl	-

*Add water to Master Mix first, TAQ last. **Change "Stock Concentration" parameters as necessary. Different reagent sources supply varying stock concentrations. $^{\pm}$Do not add DNA template until Master Mix reaction tubes have been removed from the reagent Mixing (MM) area.

Primer Sets for *R. salmoninarum* 2nd (Nested) Round Amplification

P4 (round 2 forward)	5'-AT TCT TCC ACT TCA ACA GTA CAA GG-3'
M38 (round 2 reverse)	5'-C ATT ATC GTT ACA CCC GAA ACC-3'

Gel Concentration		**Weight of agarose (grams)**	**Volume of Buffer (ml)**

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

D. Worksheet D - Photodocumentation of the PCR Product Gel

Case Number _____ Date:

Samples

Affix gel photo documentation to this sheet and make notes on results:

AFFIX PHOTODOCUMENTATION

Notes:

Chapter 4

Virology

4.1 Introduction

The procedures described in this chapter are specifically for the detection of Infectious Hematopoietic Necrosis Virus (IHNV), Infectious Pancreatic Necrosis Virus (IPNV), Infectious Salmon Anemia Virus (ISAV), Largemouth Bass Virus (LMBV), *Oncorhynchus masou* Virus (OMV), Spring Viremia of Carp Virus (SVCV), Viral Hemorrhagic Septicemia Virus (VHSV) and White Sturgeon Herpesvirus (WSHV).

The initial detection method for all of these viruses is by observing cytopathic effect (CPE) in cell culture using virus isolation procedures. The presence of IHNV and VHSV may be confirmed using either serum neutralization or polymerase chain reaction (PCR) techniques. The presence of IPNV and SVCV is confirmed by serum neutralization. The presence of LMBV and ISAV is confirmed using PCR. OMV and WSHV suspect cultures will be sent to an appropriate laboratory for confirmation.

These procedures may also detect other replicating agents not listed here. When this occurs, every attempt will be made to complete the identification of the organism. Some of these viruses may occur in combination and the finding of one agent will not preclude following procedures that may identify other agents.

If one of these viruses or an unknown replicating agent is found, the proper parties and authorities will be notified in a timely manner and at least one representative sample of each isolate should be archived at -70°C to be used for future reference.

Blind passage of samples not exhibiting CPE after 14 days of primary incubation is included in these procedures to determine if it provides a significant increase in detection of viral agents. It is requested that laboratories using these procedures summarize their findings of primary and blind passage detections by virus and provide it to the Handbook Revision and Oversight Committee annually. If the data shows that blind passage of these samples is not providing a sufficient increase in viral detection, it will be removed from the procedures.

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U.S. Fish and Wildlife Service, the United States government, and/or the American Fisheries Society. Any comparable instrument, laboratory supply or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

4.2 Selection of Appropriate Cell Lines

Selection is based on the ability of the cell lines to detect the viruses of interest and, whenever possible, utilizing cell lines capable of detecting multiple different viral agents to increase the efficiency of the laboratory procedures. At the minimum, one cell line with high sensitivity for the virus of interest will be for all samples and at least two cell lines should be used to maximize the detection of viral agents present in the samples. (General references: Bouchard, 1999; OIE, 2000; Plumb, 1999b; Thoeson, 1994; Wolf, 1988)

A. General Considerations

1. All viral testing will utilize cell lines obtained directly from the American Type Culture Collection (ATCC) when available.

2. At the minimum, all cell lines should be tested annually for viral sensitivity and mycoplasma infection.

3. Work with only one cell line at a time.

4. Aseptic technique is required for cell culture work.

B. Cell Line Sensitivities

1. The EPC cell line provides high sensitivity for IHNV, SVCV and VHSV.

2. The SHK-1 cell line provides high sensitivity for ISAV.

3. The FHM and BF-2 cell lines both provide high sensitivity for LMBV.

4. CHSE-214 cell line provides high sensitivity for IPNV and OMV.

5. The WSS-2 cell line provides high sensitivity for WSHV.

Table 4.1 - Recommended cell lines to detect target viruses

Virus[a]	Cell Line	Common Name	ATCC[b] Designation
IHNV	Epithelioma Papulosum Cyprini	EPC	
IPNV	Chinook Salmon Embryo	CHSE-214	CRL-1681
ISAV	Salmon Head Kidney	SHK-1	
LMBV	Fat Head Minnow	FHM	CCL-42
	or Bluegill Fry	BF-2	CCL-91
OMV	Chinook Salmon Embryo	CHSE-214	CRL-1681
SVCV	Epithelioma Papulosum Cyprini	EPC	
VHSV	Epithelioma Papulosum Cyprini	EPC	
WSHV	White Sturgeon Spleen	WSS-2	

[a] Viruses: IHNV- Infectious Hematopoietic Necrosis Virus; IPNV- Infectious Pancreatic Necrosis Virus and other related birnaviruses; ISAV – Infectious salmon anemia virus; LMBV – Largemouth Bass Virus and other related iridoviruses; OMV - *Oncorhynchus masou* Virus; SVCV – Spring viremia of carp; VHSV - Viral Hemorrhagic Septicemia Virus; WSHV - White Sturgeon Herpesvirus.
[b] American Type Culture Collection, Rockville, MD Certified Cell Lines

4.3 Cell Culture

Standard animal cell culture techniques are used with adaptations for fish cell lines when necessary i.e., incubation temperature. Normal appearing cultures composed of rapidly dividing cells will be used for all assay work. Cells will be routinely subcultured to maintain healthy cells, approximately once every two weeks for most cell lines, or split weekly for seeding plates. Aseptic technique is required when working with any cell line. Only one cell line is worked with at a time. (General references: Freshney, 1983; Jakoby, 1979; Merchant, 1964; Rovozzo, 1973; True, 2000)

A. Subculture Procedures for Flasks

1. Suggested split ratios and seeding rates are given in Table 4.2.

2. Remove tissue culture medium by decanting off fluid.

3. Slowly add Trypsin-Versene (EDTA) (4.9.E) solution and rock the flask gently for 1 minute. In a 75 cm^2 flask a volume of 3-4 mls is sufficient to coat cells.

4. Decant again.

5. Carefully observe the cell layer and repeat steps 4.3.A.3 and 4 as necessary until cells start detaching from flask. Dislodging of the cells may be completed by sharply striking the edge of the flask against the heel of a hand.

6. Add tissue culture medium to neutralize the trypsin. In a 75 cm^2 flask, 10 mls is sufficient.

7. Triturate to break up cell clumps and add an appropriate volume of fresh tissue culture medium for transferring to other flasks. Enumeration of cells in the suspension may be done at this time to determine the necessary volume to transfer. (4.A2)

8. Aspirate and dispense into new flasks. The sub-cultivation ratio is generally 1:4 to 1:6. Following manufacturers recommendations, bring total volume in each new flask up to the acceptable level with the appropriate tissue culture medium. For a 75 cm^2 flask this will usually be about 20 mls. MEM-10/Hepes (4.9.G) works well in an open system for all cell lines listed in Table 4.2 except SHK-1 which does best with L-15 (4.9.H).

9. Incubate flasks at room temperature (20-25°C) until they reach confluence and then incubate at the appropriate temperature for that cell line.
    a. SHK-1 and CHSE-214 cells should be held at 15°C;

    b. WSS-2 cells should be held at 20°C;

    c. FHM and BF-2 cells should be held at 25°C;

    d. EPC cells may be held at 15-25°C.

Table 4.2 - Seeding Guidelines for the Subculture of Fish Cell Lines

CELL LINE		Suggested	INCUBATION TEMP (°C)	
Common Name	Nominal split Ratio	Seeding Rate (per cm^2)	Suggested	Range
BF-2	1:2 - 3	100,000	25 – 30	20 - 30
CHSE-214	1:3 - 6	100,000	15 - 20	4 - 27
EPC	1:3 - 6	250,000	15 - 25	15 - 30
FHM	1:4 - 6	250,000	25 - 30	0 - 36
WSS-2	1:4 - 8	150,000	20 - 25	20 - 30
SHK-1	1:2 - 3	250,000	15 - 20	15 - 20

B. Seeding Procedures for Plates

1. Monolayers on plates are prepared approximately 24 to 48 hours prior to inoculation with the sample.

2. A flask of visually healthy cells approximately 7-10 days old is selected and trypsinized as described previously. After neutralizing the trypsin with tissue culture medium, the total volume in the flask is adjusted to provide a cell concentration appropriate for the seeding rate listed in Table 4.2 and the area of the wells to be seeded. The appropriate volume of the cell suspension is then pipetted into each well of the plate.

   Example: When seeding EPC cells in a 24 well plate, 0.5 mls of a 1 X 10^6 cells per ml suspension is dispensed per well.

3. At a minimum, control wells are included in each plate set and should be included on each plate whenever possible. Control wells are made by dispensing tissue culture medium into plate wells that contain normal looking cell monolayers that have not been inoculated with a sample. These wells are observed during the incubation along with the sample wells for abnormalities that may arise due to media or cell problems. A plate set refers to the group of plates seeded from a single flask at the same time.

4. Incubate plates overnight at room temperature (approximately 20-25°C).

4.4 Sample Processing Procedures

Tissue processing for viral culture is described below. Ingredients and preparation procedures for buffers and other solutions/media are listed in section 4.9. (General references: Amos, 1985; OIE, 2000; Rovozzo, 1973; True, 2000; Wolf, 1988)

A. General Considerations

1. As during sampling and transport, care is taken to protect tissues and fluids from exposure to UV light and temperatures lethal to the viruses of interest.

2. During sample processing, dilution levels are selected from the acceptable range to maintain maximal virus titers and minimize cell toxicity.

3. Following inoculation of test media, remaining tissue/fluid products are kept at 4°C until all assays are completed. Subsequent to the completion of the assays, all material is decontaminated and discarded.

B. Processing of Kidney and Spleen Samples

1. If transport medium (2.3.C.2) is used, it is poured off and disinfected before discarding. Tissue samples are weighed to the nearest 0.1 g and sterile sample dilution medium (4.9.A) is added to make a dilution of 1:10 to 1:100 (w/v). Unless there is a high potential for cell toxicity from the sample, the 1:10 dilution will be used.

2. Tissues are homogenized and a measured amount of homogenate is pipetted into a sterile tube for centrifugation.

3. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 times gravity relative centrifugal force (X g).

4. An aliquot of supernatant (for virologic evaluation) is transferred to a tube containing an equal amount of antibiotic incubation medium (anti-inc) (4.9.B or 4.9.C). Sample dilution is now 1:20 volume/volume.

Tubes are vortexed and incubated for 2 hours at 15°C or 12-24 hours at 4°C. Samples are then re-centrifuged at 2000-3000 X g and the supernatant is inoculated onto tissue culture plates as described in the inoculation procedures (4.5.A).

C. Processing of Coelomic (Ovarian) Fluid Samples

1. An equal volume from each ovarian fluid sample is pipetted into a sterile tube for centrifugation.

2. Centrifuge the tubes at 2000-3000 X g for 15 minutes at 4°C.

3. Undiluted ovarian fluid may be used to inoculate cell cultures as described in the inoculation procedures or up to a 1:5 dilution (1 part ovarian fluid to 4 parts anti-inc)(4.9.B or 4.9.C) may be made.

4. If a dilution is made, tubes are vortexed and incubated for 2 hours at 15°C or 12-24 hours at 4°C. Samples are then re-centrifuged at 2000-3000 X g and the supernatant is inoculated onto tissue culture plates as described in the inoculation procedures.

4.5 Screening Method for Viral Isolation

The initial detection or screening method used for all listed viral agents is the observation of CPE in cell culture. Standard viral propagation techniques include plate inoculation and incubation for a minimum of 14 days with re-inoculation of samples exhibiting CPE. Blind passage of samples not exhibiting CPE is included to optimize detection of low titer and/or slow growing viruses. To maximize detection of viral agents, samples should be inoculated on at least two different cell lines. (General references: Amos, 1985; OIE, 2000; Bouchard, 1999; Plumb, 1999b; Rovozzo, 1973; True, 2000; Wolf, 1988)

A. Plate Inoculation Procedures for Primary Culture

1. General Considerations
    a. All cell monolayers to be inoculated are to be at least 80% confluent, approximately 24 hours old, and visually healthy.

    b. Tissue culture plates are identified by labeling with the cell line, date of inoculation, and sample information.

    c. Aseptic technique is required.

2. Tissue culture medium is decanted from plates.

3. Inoculate with replication at least 2 cm^2 of cell monolayer with a minimum of 100µl from each sample.

    Example: If using 24 well plates (2 cm^2/well), 100µl of each sample would be inoculated onto each of two wells of the plate.

To allow for viral adsorption, incubate plates for 1 hour with gentle rocking at least every 15 minutes or continuously on a laboratory rocker.
    a. Incubation temperature for IPNV, IHNV, VHSV, ISAV, and OMV is 15°C.

    b. Incubation temperature for WSHV, SVCV, and LMBV is 20-25°C.

5. Dispense an adequate volume of the appropriate tissue culture medium into each well of the plate. MEM-5/Hepes (4.9.F) works well in an open system for all cell lines listed in Table 4.2 except SHK-1 which does best with Leibovitz's L-15 (4.9.H). If using 24 well plates, 0.5 ml of media is adequate.

6. Seal each plate.

7. Incubate plates at the appropriate temperature for a minimum of 14 days.
    a. Incubate for IHNV, IPNV, ISAV, OMV, and VHSV at 15°C.

    b. Incubate for WSHV at 20°C.

c. Incubate for LMBV and SVCV at 20-25°C.

8. Monitor cells at least twice per week for cytopathic effect (CPE). CPE is defined as any morphological change that cells may demonstrate in response to viral or toxic agents. It may range from foaming of the cytoplasm to focal clumping or local destruction of cells. Examples of the appearance of normal cell line monolayers and the CPE typical of these viruses are shown in Figures 4.1-4.12.

9. Re-inoculations are made from representative wells exhibiting CPE on these primary inoculations and from at least one well of all samples not exhibiting CPE (blind passage) according to the procedure in 4.5.B.

B. Re-inoculation Procedure

1. General Considerations:
   a. Re-inoculation of wells showing toxicity and to confirm the presence of virus with typical CPE may be performed on individual wells at any time during the primary incubation. These will be maintained as individual samples and plated with replication during the re-inoculation procedure.

   b. Blind passage from at least one well of all samples not exhibiting CPE will be performed after 10-14 days of incubation of the primary culture. It is suggested that the wells remaining on this plate be left intact and observed for at least another 7-10 days for a total initial incubation period of 21 days. These samples may be combined in up to a 5 pool sample (representing up to 25 fish) and plated with replication during the re-inoculation procedure.

      Example: On a lot inspection using 5 fish pools and 24 well plates, 2 of the 12 samples exhibit CPE at day 5 and re-inoculation is performed as described below. The remaining 10 samples (20 wells representing 50 fish) show no evidence of CPE after 14 days of primary incubation and re-inoculation is performed by combining one well from each of 5 samples and inoculating this pooled sample onto 2 wells of a 24 well plate. This is repeated with the other 5 samples using a total of 4 wells on the re-inoculation plate.

   c. As in the initial tissue processing, sample dilution levels for re-inoculation are selected from the acceptable range to maintain maximal virus titers and minimize cell toxicity.

   d. Aseptic technique is required.

2. Using a pipette, stir and scrape the bottom of the well to be subcultured to dislodge the cell layer.

3. Aspirate the fluid and cell debris from the well and place in a sterile tube for centrifugation.

4. Tubes are centrifuged at 4°C for 15 minutes at 2000-3000 X g.

5. Remove a measured amount of supernatant and place in a separate sterile tube for dilution.

6. Dilute Samples:
    a. For wells exhibiting CPE, use the lowest dilution possible for re-inoculation not to exceed 1:100 using sample dilution medium (4.9.A).

    b. For blind passage samples, up to a 1:5 dilution may be made.

7. If bacterial or fungal contamination is present, the sample should be filtered through a 0.45µ filter before inoculation onto the plate.

8. Use the appropriate amount of each of these solutions to inoculate a new plate as described in the Plate Inoculation Procedure (4.5.A).

9. Monitor these re-inoculation plates at least twice per week for CPE. Total incubation time for both the primary and re-inoculation or blind pass samples is 28 consecutive days.

> **Example:** If the blind pass is performed on day 14 of the primary incubation, the re-inoculation plate is observed for at least an additional 14 days. If the blind pass is performed on day 10, the re-inoculation plate is observed for at least 18 days.

10. Results:
    a. **If no CPE is noted after the 28 day combined incubation period with no apparent problems in the assay, samples are reported as negative and may be discarded using the proper decontamination procedures.**

    b. If CPE occurs at any time during this assay, it is considered a **PRESUMPTIVE positive** result and the identification of the virus should be confirmed by the appropriate method.

4.6 Identification of Viruses

Methods used for confirmation must have high specificity for the agents they are used to identify. Serum neutralization has long been used as a standard for viral identification and Polymerase Chain Reaction (PCR) procedures have recently been developed for many of the listed viruses. Although other methods exist to identify some of the listed viruses, these two methods have been selected for confirming the presence of all of these agents except OMV and WSHV. The identification of IHNV, VHSV, IPNV and SVCV may be confirmed using serum neutralization. The identification of IHNV, VHSV, LMBV, and ISAV may be confirmed by PCR. OMV and WSHV suspect cultures will be sent to an appropriate reference laboratory for confirmation.

A. Infectious Hematopoietic Necrosis Virus (IHNV) - Infectious Hematopoietic Necrosis Virus (IHNV) is an enveloped bullet shaped single stranded RNA virus belonging to the Novirhabdovirus genus of the Rhabdoviridae family. IHNV has a wide geographic range that includes North America, Europe and the Far East. The virus is primarily found in salmonids with rainbow trout fry being highly susceptible to disease. Older fish are more resistant to infection but may become carriers. Transmission is primarily horizontal but cases of egg associated transmission have been recorded as well as transmission by fomites. The virus may be shed in ovarian fluid and excretory products such as feces and may also be isolated from the kidney, spleen, encephalon, and digestive tract of clinically ill fish. Under natural conditions, most clinical disease from IHNV is seen in fry when water temperature is between 8-15°C with fish exhibiting darkening of the skin, ascites, exophthalmia, and petechial hemorrhages internally and externally. Degeneration and necrosis of the hematopoietic tissue in the kidney is thought to be the actual cause of mortality. (Egusa, 1991; OIE, 2000; Wolf, 1988)

Screening method:

a. Cell culture on EPC cell line incubated at 15°C.

b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for IHNV.**

d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.
    i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers

are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. Polyethylene glycol (PEG) has been used to aid in the visualization of plaque formation but is not necessary to detect IHNV. (Batts, 1989) See Figures 4.1 and 4.2.

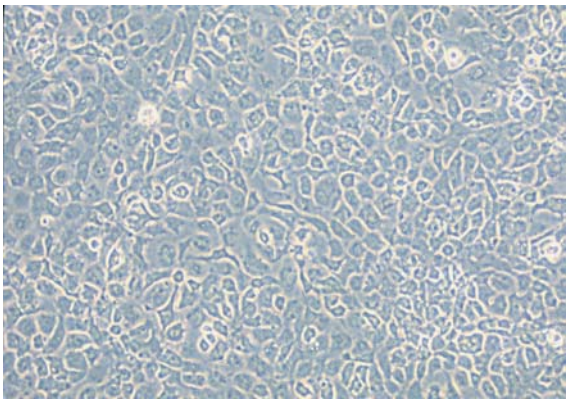


Figure 4.1 Normal EPC monolayer
Photo Courtesy of Jim Winton, USGS

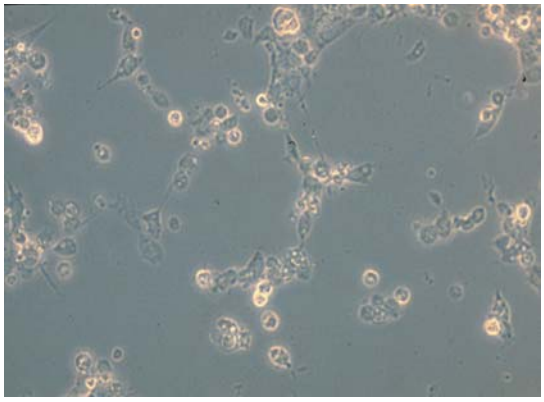


Figure 4.2 CPE typical of a rhabdovirus on EPC monolayer
Photo Courtesy of Jim Winton, USGS

ii. The methods below may be used to confirm that the cause of the CPE is due to the presence of IHNV.

2. Confirmation methods for IHNV:
   a. Serum Neutralization method: See section 4.7 for the general procedure.
      i. Use the cell line on which the initial CPE was produced.

      ii. Incubate plates at 15°C

   b. Polymerase Chain Reaction (PCR) method for confirmation of IHNV (Modified from Arakawa, 1990): The Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.
      i. Extraction of RNA from cell culture fluid (Heat RNA release method)
         1. Dilute cell culture fluid 1:50 in molecular grade RNase free water by adding 2 µl fluid to 98 µl water in microcentrifuge tubes.

         2. Heat tubes to 95°C for 2 min in a water bath, heat block, or thermocycler.

3. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself)

4. Quantify RNA Template in the spectrophotometer. The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µl or use up to 5µl/reaction if reading falls below 50 ng/µl.

ii. Production of DNA by Reverse Transcription and Amplification by First Round PCR

1. QA/QC (see Chapter 6 for Specific QA/QC considerations for PCR)

2. Using Worksheet A "PCR Sample Data/Log Sheet" (Appendix 4.A1), record appropriate data for each sample to be tested by PCR.

3. Using Worksheet B "Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens" (Appendix 4.A1) record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls).

4. First Round Primers for IHNV:
   a Forward: 5'-TCA AGG GGG GAG TCC TCG A-3'

   b Reverse: 5'-CAC CGT ACT TTG CTG CTA C-3'

5. First Round Thermocycler Program for IHNV
   a Pre-dwell at 50°C for 15 minutes

   b Preheat or "Jumpstart" sample to 95°C for two minutes.

   c 25 cycles as follows:
      i. Denaturing at 95°C for 30 seconds.
      ii. Annealing at 50°C for 30 seconds.
      iii. Extending at 72°C for 60 seconds.

   d Post dwell at 72°C for 7 minutes.

   e Hold samples at 4°C after cycling is complete.

iii. "Nested" Second Round PCR for IHNV

1. QA/QC (see Chapter 6 for Specific QA/QC considerations for PCR)

2. Again use Worksheet B for the Second Round (Appendix 4.A1) to record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples and controls to be processed.

3. Second Round Primers for IHNV:
   a Forward: 5'-TTC GCA GAT CCC AAC AAC AA-3'

   b Reverse: 5'-GCG CAC AGT GCC TTG GCT-3'

4. Second Round Thermocycler Program for IHNV
   a. Preheat or "Jumpstart" sample to 95°C for two minutes.

   b. 25 cycles as follows:
      i. Denaturing at 95°C for 30 seconds.
      ii. Annealing at 50°C for 30 seconds.
      iii. Extending at 72°C for 60 seconds.

   c. Post dwell at 72°C for 7 minutes.

   d. Hold samples at 4°C after cycling is complete.

   PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

iv. Visualization of PCR Product by Electrophoresis (See 6.3.C)
   1. Visualize the DNA - Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays.
      a. **Bands occurring at the 786 bp location in the First Round Assay and the 323 bp location in the Second Round Assay are confirmatory for IHNV and are reported as POSITIVE.**

      b. **The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for IHNV.**

   2. Photograph the gel (6.3.G)- **Photo document all gels** and attach the photo to the case history information. (Worksheet C "Photodocumentation of the PCR Product Gel" Appendix 4.A1)

B. Infectious Pancreatic Necrosis Virus (IPNV) - Infectious Pancreatic Necrosis Virus (IPNV) is a nonenveloped icosahedral shaped bi-segmented double-stranded RNA virus belonging to the Aquabirnavirus genus of the Birnaviridae family. There are many viruses in the Aquabirnavirus group most of which have not been shown to cause disease in fish so care must be taken to confirm that the virus present is IPNV. IPNV has a wide geographic range that includes North and South America, Asia, and Europe. It is very stable under a wide range of environmental conditions and is capable of surviving for several days in both fresh and saltwater. It is resistant to a wide range of chemical disinfectants including ether, chloroform, and quaternary ammonium compounds but is deactivated by 70% ethanol. Isolates display wide antigenic diversity and virulence. There are two sero-groups that do not cross-react in serum neutralization tests with the majority of strains belonging to sero-group A. IPNV has been isolated from several species of marine and freshwater fish and shellfish. Acute catarrhal enteritis has primarily been seen in salmonid fry and fingerlings with initial mortality occurring in the more robust individuals. Fish that survive the disease may become asymptomatic carriers and shed the virus through the feces and sex products. IPNV may be transmitted vertically as well as horizontally. (Bruno, 1996; Egusa, 1991; OIE, 2000; Roberts, 1982; Wolf, 1988)

1. Screening method:
    a. Cell Culture on CHSE-214 cell line incubated at 15°C.

    b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

    c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for IPNV.**

    d. If CPE typical of IPNV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive**.
        i. The appearance of CPE typical of IPNV is described as stellate shaped plaques with spindle-shaped cells. Some of the cells within the plaque will exhibit nuclear pyknosis (nuclei shrink in size and chromatin condenses) with other cells appearing normal. See figures 4.3 and 4.4. Typically, positive cultures result in rapidly lytic CPE but some cells may survive and reform a normal looking monolayer.

        ii. The serum neutralization method is used to confirm the cause of the CPE is due to the presence of IPNV.

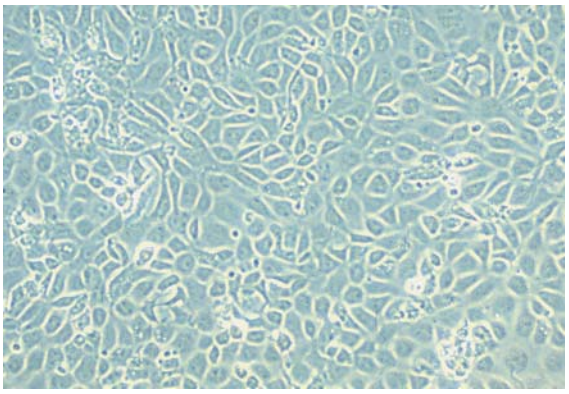


Figure 4.3 Normal CHSE-214 monolayer
Photo Courtesy of Jim Winton, USGS

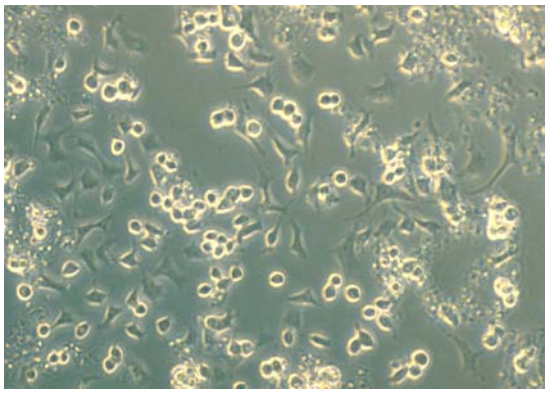


Figure 4.4 CPE typical of IPNV on CHSE-214 cells
Photo Courtesy of Jim Winton, USGS

2. Confirmation method for IPNV - Serum Neutralization method: See section 4.7 for the general procedure.
    a. Use the cell line on which the initial CPE was produced.

    b. Incubate plates at 15°C

C. Infectious Salmon Anemia Virus (ISAV) - Infectious Salmon Anemia Virus (ISAV) is a spherical enveloped single-stranded RNA virus most likely belonging to the Orthomyxovirus genus of the Orthomyxoviridae family. Disease is mostly seen in Atlantic salmon in salt water in the spring and fall associated with rapidly changing water temperature. Characteristics of the disease include anemia, ascites, petechial hemorrhages on the peritoneal surface and perivisceral fat, and congestion of the liver, spleen, kidney and upper digestive tract. The virus has been isolated from Atlantic salmon, Rainbow and Brown or sea trout, and Atlantic herring from Europe and the Atlantic coast of North America. (Bruno, 1996; OIE, 2000)

1. Screening method:
    a. Cell Culture on SHK-1 cell line incubated at 15°C. (Bouchard, 1999)

    b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

    c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for ISAV.**

    d. If CPE typical of ISAV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive.**
        i. The appearance of CPE typical of ISAV is described as plaques of vacuolated cells that round up and loosen from the growth surface. It

may progress to involve the entire cell sheet with only small rounded, refractile and necrotic cells observable. See Figures 4.5 and 4.6.

ii. The Polymerase Chain Reaction (PCR) method is used to confirm the cause of the CPE is due to the presence of ISAV.

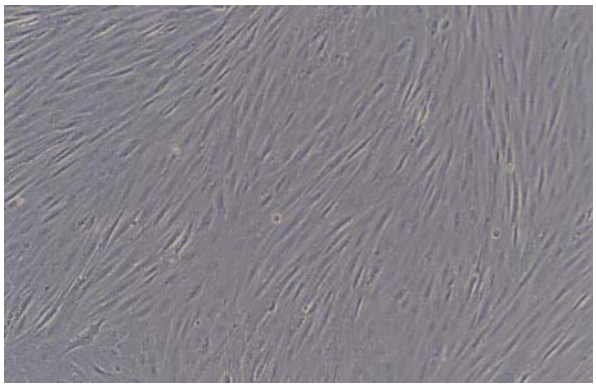


Figure 4.5 Normal SHK-1 monolayer
Photo Courtesy of Jim Winton, USGS

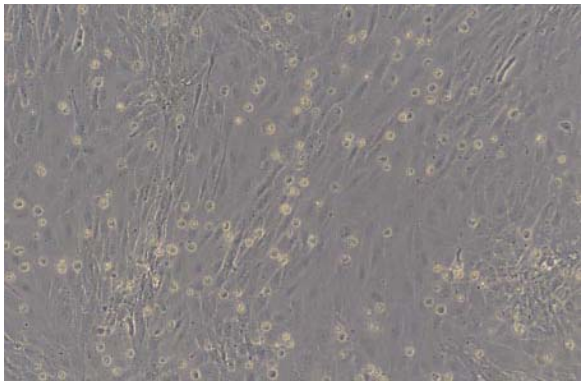


Figure 4.6 CPE typical of ISA on SHK-1 cells
Photo Courtesy of Jim Winton, USGS

2. Confirmation method for ISAV - Polymerase Chain Reaction (PCR) (Modified from Bouchard, 1999) - The Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.
    a. Extraction of RNA from Cell Culture Fluid (Heat RNA Release method)
        i. Dilute Cell Culture fluid (with some cell scrapings) 1:50 in molecular grade RNAase free water by adding 2 µl fluid to 98 µl water in microcentrifuge tubes.

        ii. Heat to 95°C for 2 min in a heat block, water bath, or thermocycler.

        iii. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from reannealing on itself)

        iv. Quantify RNA Template in the spectrophotometer. The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µl or use up to 5µl/reaction if reading falls below 50 ng/µl.

    b. Formation of DNA by Reverse Transcription and Amplification by PCR
        i. QA/QC (see 6.2 for Specific QA/QC considerations for PCR)

ii. Using Worksheet A "PCR Sample Data/Log Sheet" (Appendix 4.A1), record appropriate data for each sample to be tested by PCR.

iii. Using Worksheet B "Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens" (Appendix 4.A1) record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls and empty slots in the assay).

iv. Primers for ISAV:

1. Forward: 5'-GGC TAT CTA CCA TGA ACG AAT C-3'

2. Reverse: 5'-TAG GGG CAT ACA TCT GCA TC-3'

v. Thermocycler Program for ISAV

1. Pre-dwell at 42°C for 15 minutes

2. Preheat or "Jumpstart" sample to 94°C for five minutes.

3. 40 cycles as follows:

a. Denaturing at 94°C for 45 seconds.
b. Annealing at 59°C for 45 seconds.
c. Extending at 72°C for 105 seconds.

4. Post dwell at 72°C for 7 minutes.

5. Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

c. Visualization of PCR Product by Electrophoresis (See 6.3.C)

i. Visualize the DNA - Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in the PCR assays.

1. **A band occurring at the 493 bp location is confirmatory for ISAV and the sample is reported as POSITIVE.**

2. **The lack of the appropriate band with no indication of problems with the assay are reported as NEGATIVE for ISAV.**

ii. Photograph the gel (6.3.G)- **Photo document all gels** and attach the photo to the case history information. (Worksheet C "Photodocumentation of the PCR Product Gel" Appendix 4.A1)

D. Largemouth Bass Virus (LMBV) - Largemouth Bass Virus (LMBV) is an icosahedral enveloped double-stranded DNA virus in the Ranavirus genus of the Iridoviridae family. LMBV infection has been found in centrarchid and ecocid populations in the Mid-West and Southeastern United States and has been found experimentally to be associated with mortality in juvenile largemouth bass. (Plumb, 1999a) During an active infection, the virus may be isolated from several tissues including the kidney, spleen, and swim bladder.

1. Screening method:
    a. Cell culture on FHM or BF-2 cell lines incubated at 20-25°C. (Plumb, 1999b)

    b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

    c. Re-inoculations are made from representative wells exhibiting CPE on the initial inoculations and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for LMBV.**

    d. If CPE typical of LMBV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive.**
        i. The appearance of CPE typical of LMBV is described as circular cell free areas with rounded cells at the margins. See Figures 4.7 through 4.10.

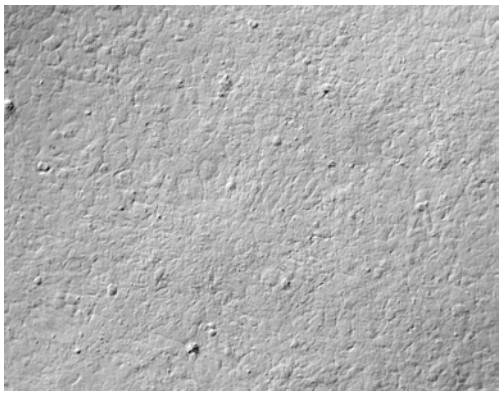


Figure 4.7 Normal FHM monolayer Photo Courtesy of John Grizzle, Auburn University

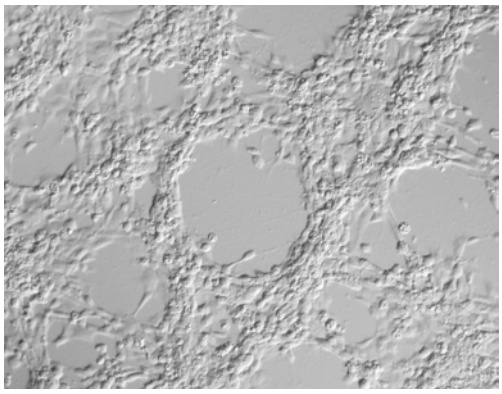


Figure 4.8 CPE typical of LMBV on FHM cells. Photo Courtesy of John Grizzle, Auburn University

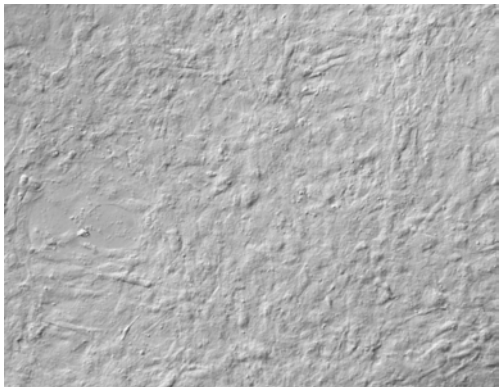


Figure 4.9 Normal BF-2 monolayer Photo Courtesy of John Grizzle, Auburn University

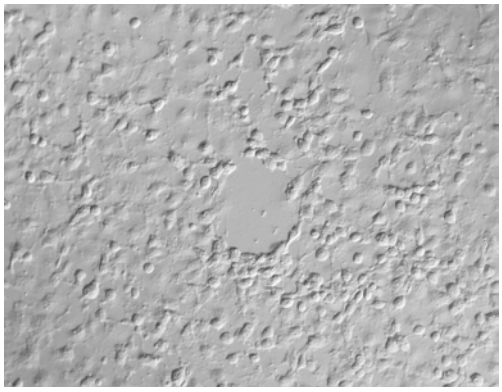


Figure 4.10 CPE typical of LMBV on BF-2 cells. Photo Courtesy of John Grizzle, Auburn University

ii. The Polymerase Chain Reaction (PCR) method is used to confirm that the cause of the CPE is due to the presence of LMBV.

2. Confirmation method for LMBV - Polymerase Chain Reaction (PCR) (Modified from Plumb, 1999b)–This is a DNA containing virus so DNA is extracted from cell culture fluid and amplified with forward and reverse primers. The DNA products are then visualized by agarose gel electrophoresis.
    a. Extraction of DNA from cell culture
        i. Supernatant and cells from suspect sample wells are removed and cells lysed in 1ml of 50mM KCL, 10 mM tris-HCL at pH 9.3, and 3 mM Mg Cl_2 containing 0.5% Tween 20.

        ii. Transfer 500µl of lysate to a microcentrifuge tube.

        iii. Add proteinase K solution (5.6.E) to a final concentration of 100µg/ml (example: if stock solution is 20 mg/ml, add 2.5 µl).

        iv. Incubate overnight at 37°C.

        v. Add 500µl of 70% phenol-40% chloroform and vortex.

        vi. Centrifuge at 3-5,000 Xg to separate layers.

        vii. Pipet out the aqueous (top) layer and place in a clean centrifuge tube being careful not to contaminate with any material from the phenol-chloroform layer.

        viii. Repeat steps v.-vii.

        ix. Add to the aqueous layer after the second extraction 50µl of potassium acetate and 1 ml of absolute ethanol. Mix gently.

x. Hold the sample at -20°C for at least 10 minutes to precipitate the DNA.

xi. Centrifuge the sample at 3-5,000 Xg for 30 minutes.

xii. Decant the alcohol solution, add 500µl of 70% ethanol, and mix by inverting the tube.

xiii. Centrifuge at 3-5,000 Xg for 5 minutes and decant off the alcohol.

xiv. Resuspend the DNA pellet in 200µl of TE buffer (10 mM tris-HCL at pH 8.0 and 1mM EDTA).

b. Amplification of LMBV DNA:

i. General QA/QC Considerations (see 6.2 for Specific QA/QC considerations for PCR):

ii. Using Worksheet A "PCR Sample Data/Log Sheet" (Appendix 4.A1), record appropriate data for each sample to be tested by PCR.

iii. Using Worksheet B "Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens" (Appendix 4.A1) record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls in the assay).

iv. Primers for LMBV:

1. Forward: 5'-GAC TTG GCC ACT TAT GAC-3'

2. Reverse: 5'-GTC TCT GGA GAA GAA GAA-3'

v. Thermocycler program for LMBV

1. Pre-dwell sample to 94°C for 5 minutes.

2. 30 cycles of the following regime

a. Denaturing at 94°C for 60 seconds.
b. Annealing at 45°C for 60 seconds.
c. Extending at 60°C for 120 seconds.

3 Post dwell at 72°C for 2 minutes.

4 Hold samples at 4°C after cycling is complete.

PCR Products can be refrigerated for one month or frozen at -70 ° C for long-term storage.

c. Visualization of PCR Product by Electrophoresis (6.3.C) - Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated location according to primers used.
    i. **A band occurring at the 495 bp location is confirmatory for LMBV and the sample is reported as POSITIVE.**

    ii. **The lack of the appropriate band with no indication of problems with the assay are reported as a NEGATIVE sample for LMBV.**

d. Photograph the gel (6.3.G) - **Photo document all gels** and attach the photo to the case history information. (Worksheet C "Photodocumentation of the PCR Product Gel" Appendix 4.A1)

E. Oncorhynchus Masou Virus (OMV) -

Oncorynchus Masou Virus (OMV) is an enveloped double-stranded DNA virus belonging to the Herpesvirus genus of the Herpesviridae family. Salmonids are the only fish known to be susceptible to infection with OMV with kokanee being the most susceptible. The geographic range has so far been limited to Japan and Eastern Asia. The initial disease is a septicemia that may cause edema and hemorrhage in fry during which time virus will be shed in the feces and urine and isolated from the liver, kidney, and spleen. Several months later, survivors may develop epithelial tumors around the mouth and fins with virus being easily isolated from these lesions. Most disease is seen in water temperatures below 14 °C and although OMV may be isolated from ovarian fluid at spawning, as long as eggs are disinfected after fertilization, transmission is by the horizontal route. (OIE, 2000; Wolf, 1988)

1. Screening method:
    a. Cell culture on CHSE-214 cell line incubated at 15°C.

    b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

    c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for OMV.**

    d. If CPE typical of OMV is produced at any time during the incubation, the sample is considered **PRESUMPTIVELY positive**.
        i. The appearance of CPE typical of OMV is described as the formation of rounded cells which progress to marked syncytia and eventual lysis of the entire cell sheet. See Figures 4.11 and 4.12.

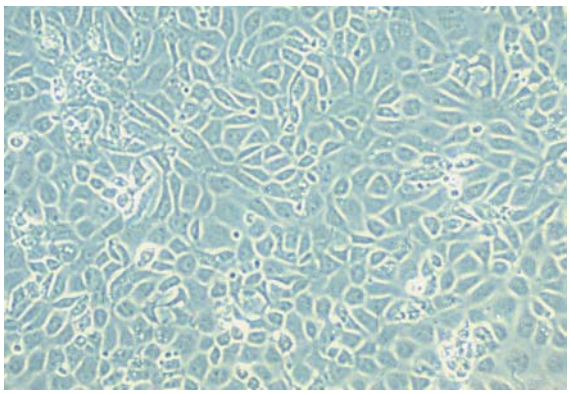


Figure 4.11 Normal CHSE-214 monolayer
Photo Courtesy of Jim Winton, USGS

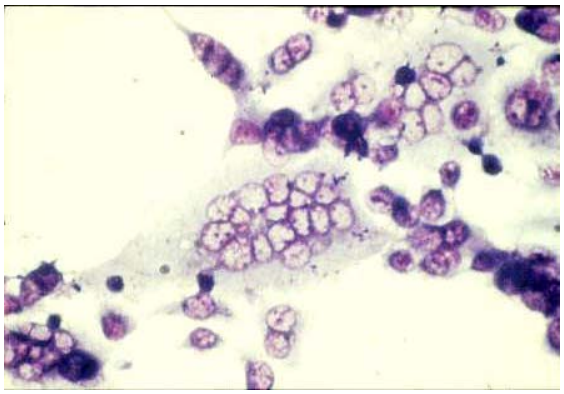


Figure 4.12 CPE typical of OMV on CHSE-214 cells
Photo Courtesy of Mamoru Yoshimizu, Hokkaido University

ii. Suspect samples are sent to an appropriate laboratory for confirmation that the CPE is due to the presence of OMV.

2. Confirmation method:
   a. OMV is considered an exotic pathogen in the United States and many regulations prohibit the maintenance of live virus for positive controls by serological methods. A PCR method will be available soon for identification of OMV but at the present time OMV suspect samples must be sent to a reference laboratory capable of confirming this virus.

   b. A laboratory capable of confirming the identity of OMV is the Laboratory of Microbiology, Hokkaido University, 3-1-1 Minato-cho, Hokodate, Hokkaido 041-0821, Japan, Phone/fax: (81.138) 40.88.10

F. Spring Viremia of Carp Virus (SVCV) - Spring Viremia of Carp Virus (SVCV) is an enveloped bullet shaped single stranded RNA virus belonging to the Vesiculovirus genus of the Rhabdoviridae family. The geographic range includes European countries that experience low water temperatures during the winter and has recently been isolated in the USA. The host range includes Pike and Cyprinids with the common carp being the principle host. It causes disease in Cyprinids of all ages characterized by hemorrhages on the skin, gills, and viscera. Mortality is usually seasonal, often most severe in the spring or early summer during rising water temperatures. SVCV may be found in ovarian fluid at spawning but transmission is primarily horizontal and may involve passive transfer by parasites such as the louse and leech. (OIE, 2000; Wolf, 1988)

1. Screening method:
   a. Cell Culture on EPC cell line incubated at 20-25°C.

   b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for SVCV**.

d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.
    i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. See Figures 4.1 and 4.2 above.

    ii. The Serum Neutralization method may be used to confirm that the cause of the CPE is due to the presence of SVCV.

2. Confirmation method:
    a. Serum Neutralization method: See section 4.7 for the general procedure.
        i. Use the cell line on which the initial CPE was produced.

        ii. Incubate plates at 20-25°C.

G. Viral Hemorrhagic Septicemia Virus (VHSV) - Viral Hemorrhagic Septicemia Virus (VHSV) is an enveloped bullet shaped single stranded RNA virus belonging to the Novirhabdovirus genus of the Rhabdoviridae family. Disease in Continental Europe is mostly seen in trout, grayling, white fish, pike and turbot and is characterized by edema and hemorrhage due to impairment of osmotic balance. Disease in North America is primarily seen in Pacific herring and pilchard but the virus has been isolated from several species of marine fish in the Pacific and Atlantic Oceans around North America and from returning adult Coho and Chinook salmon. Fry are most susceptible to disease, which usually occurs at water temperatures between 4-14°C. A carrier state may develop with fish shedding virus in the feces, urine, and sexual fluids as well as being present in the internal organs. Although present in ovarian fluid, vertical transmission has not been demonstrated with VHSV. The European and North American strains of VHSV are indistinguishable by serologic methods but may be separated by PCR methods. (OIE, 2000; Wolf, 1988)

1. Screening method:
    a. Cell culture on EPC cell line incubated at 15°C.

b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for VHSV**.

d. If CPE typical of a rhabdovirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for IHNV, SVCV, or VHSV and confirmatory tests must be completed to distinguish among these viruses.
    i. The appearance of CPE typical of a rhabdovirus is described as rounded and granular cells in grape-like clusters that may exhibit margination of nuclear chromatin (optical density of nucleoli increases and nuclear membranes appear thickened). Plaques in the confluent cell monolayers are ragged in outline and contain coarsely granular debris and rounded cells. Rounded, infected cells also accumulate at plaque margins and can be present within the plaque. See Figures 4.1 and 4.2 above.

    ii. The methods below may be used to confirm that the cause of the CPE is due to the presence of VHSV.

2. Confirmation methods for VHSV:
    a. Serum Neutralization method: See section 4.7 for the general procedure.
        i. Use the cell line on which the initial CPE was produced.

        ii. Incubate plates at 15°C

    b. Polymerase Chain Reaction (PCR) method for confirmation of VHSV (Modified from Einer-Jensen K, 1995): The Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers to amplify base pair segments of the complementary DNA (cDNA) produced in RT-PCR reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subject to reverse transcription for production of appropriate cDNA, which is then amplified with forward and reverse primers. The DNA product from the amplification is then visualized by agarose gel electrophoresis.
        i. Extraction of RNA from Cell Culture Fluid (Heat RNA Release method)
            1. Dilute Cell Culture fluid (with some cell scrapings) 1:50 in molecular grade RNase free water by adding 2 µl fluid to 98 µl water in microcentrifuge tubes.

            2. Place tubes in heat block at 95°C for 2 min.

3. Immediately cool on crushed ice (cooling the fluid quickly after heating prevents the RNA from re-annealing on itself)

4. Quantify RNA Template in the spectrophotometer. The optimum amount of RNA template should be around 100 µg/mL (or 100 ng/µL). Generally, 1µL of RNA template is sufficient per RT-PCR reaction. Dilute template if more than 300 ng/µl or use up to 5µl/reaction if reading falls below 50 ng/µl.

ii. Formation of DNA by Reverse Transcription and Amplification by First Round PCR

1. QA/QC (see 6.2 for Specific QA/QC considerations for PCR)

2. Using Worksheet A "PCR Sample Data/Log Sheet" (Appendix 4.A1), record appropriate data for each sample to be tested by PCR.

3. Using Worksheet B "Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens" (Appendix 4.A1) record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples to be processed. (Add 4 to the number of samples so that there is enough to run controls in the assay).

4. First Round Primers for VHSV:
   a Forward: 5'-TCT CTC CTA TGT ACT CCA AG-3'

   b Reverse: 5'-TTC CGG TGG AGC TCC TGA AG-3'

5. Thermocycler Program for First Round VHSV
   a. Pre-dwell at 50°C for 15 minutes

   b. Preheat or "Jumpstart" sample to 95°C for two minutes.

   c. 25 cycles as follows:
      i. Denaturing at 95°C for 30 seconds.
      ii. Annealing at 50°C for 30 seconds.
      iii. Extending at 72°C for 60 seconds.

   d. Post dwell at 72°C for 7 minutes.

   e. Hold samples at 4°C after cycling is complete.

iii. "Nested" Second Round PCR for VHSV:

1. QA/QC (see 6.2 for Specific QA/QC considerations for PCR)

2. Again use Worksheet B for the Second Round (Appendix 4.A1) to record date of assay and then calculate the amount of each reagent to go into the "Master Mix" (MM) according the number of samples and controls to be processed.

3. Second Round Primers for VHSV:
    a. Forward: 5'-ATG GGC TTC AAG GTG ACA C-3'

    b. Reverse: 5'-GTA TCG CTC TTG GAT GGA C-3'

4. Thermocycler Program for Second Round VHSV
    a. Preheat or "Jumpstart" sample to 95°C for two minutes.

    b. 25 cycles as follows:
        i. Denaturing at 95°C for 30 seconds.
        ii. Annealing at 50°C for 30 seconds.
        iii. Extending at 72°C for 60 seconds.

    c. Post dwell at 72°C for 7 minutes.

    d. Hold samples at 4°C after cycling is complete.

    PCR Products can be refrigerated for one month or frozen at -70°C for long-term storage.

iv. Visualization of PCR Product by Electrophoresis (6.3.C)

1. Visualize the DNA - Carefully record locations of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in both the first and second (nested) round PCR assays.
    a. **Bands occurring at the 950 bp location in the First Round Assay and the 558 bp location in the Second Round Assay are confirmatory for VHSV and are reported as POSITIVE.**

    b. **The lack of the appropriate bands with no indication of problems with the assay are reported as NEGATIVE for VHSV.**

2. Photograph the gel (6.3.G)- **Photo document all gels** and attach the photo to the case history information. (Worksheet C "Photodocumentation of the PCR Product Gel" Appendix 4.A1)

H. White Sturgeon Herpesvirus (WSHV) - White Sturgeon Herpesvirus (WSHV) is an enveloped icosahedral shaped double-stranded DNA virus belonging to the Herpesvirus genus of the Herpesviridae family. WSHV has been found in both feral and captive populations of sturgeon in California and Oregon. WSHV-1 has been found in juvenile cultured white sturgeon less than 10 cm. The susceptibility of other sturgeon species to WSHV-1 is not known at this time. WSHV-2 has been isolated from wild and cultured subadult and adult white sturgeon. A herpesvirus has also been isolated from shortnose sturgeon, although the relationship of this isolate to WSHV-1 or WSHV-2 has not been determined. Infected fish may present with lethargy, emaciation, excessive mucus production, fluid in the gastrointestinal tract and focal skin lesions. Horizontal transmission has been demonstrated with both WSHV-1 and WSHV-2. WSHV-2 has been isolated from ovarian fluid but vertical transmission has not been demonstrated. (LaPatra, personal communication, 2002; Plumb, 1999a)

1. Screening method:
    a. Cell culture on WSS-2 cell line incubated at 20°C.

    b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.

    c. Re-inoculations are made from representative wells exhibiting CPE on the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days. **If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for WSHV**.

    d. If CPE typical of a herpesvirus is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY positive** for WSHV.
        i. The appearance of CPE typical of WSHV and other herpesviruses includes the formation of syncytia. See Figures 4.13 and 4.14.

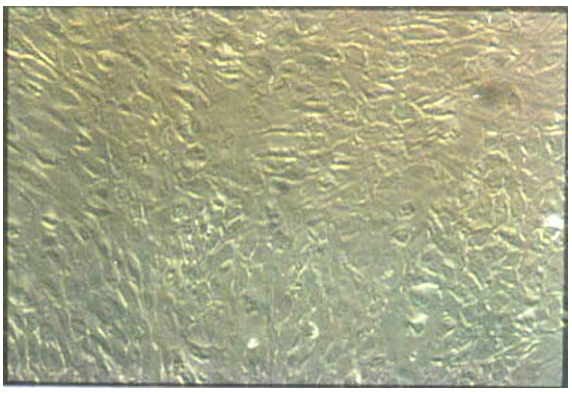


Figure 4.13 Normal WSS-2 monolayer
Photo Courtesy of Scott LaPatra, Clear Springs Foods, Inc

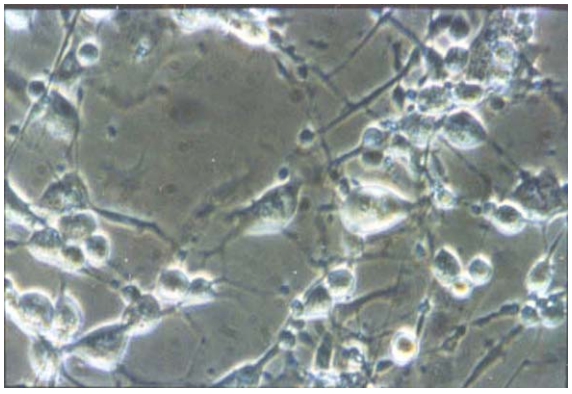


Figure 4.14 CPE typical of WSHV on WSS-2 monolayer
Photo Courtesy of Scott LaPatra, Clear Springs Foods, Inc

ii. Suspect samples are sent to an appropriate laboratory for confirmation that the CPE is due to the presence of WSHV.

2. Confirmation method:
    a. A PCR method has been developed for this virus however the necessary primers are not commercially available at this time. Therefore, suspect samples must be sent to a reference laboratory for confirmation.

    b. A laboratory capable of confirming the identity of WSHV is the Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, Phone: 530-752-3411.

4.7 Serum Neutralization

Serum neutralization or plaque reduction assays are serological methods used to confirm the identity of an unknown viral isolate. The procedures involve the use of a known dilution of specific neutralizing antiserum mixed with multiple dilutions of the homologous and suspect virus and subsequent observation of the ability of those viruses to produce CPE when inoculated onto a sensitive cell line. Normal serum from the species of animal used to produce the antiserum (usually rabbit or goat) is used as the negative control to account for nonspecific inhibitors of the virus.

A. Plate Preparation

1. Seed plates (4.3.B) with the appropriate cell line (4.2) 24-48 hours before inoculation with virus.

2. Monolayers should be visually healthy and at least 80% confluent at the time of inoculation.

B. Virus Sample Preparation

1. A dilution of neutralizing antiserum (polyclonal or monoclonal) should be used that allows neutralization of 10^3 to 10^6 plaque-forming units (PFU) or 50% tissue culture infective dose ($TCID_{50}$) per ml of the homologous virus.

2. Dilute the suspect sample and positive control virus to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} in sterile HBSS (4.9.A).

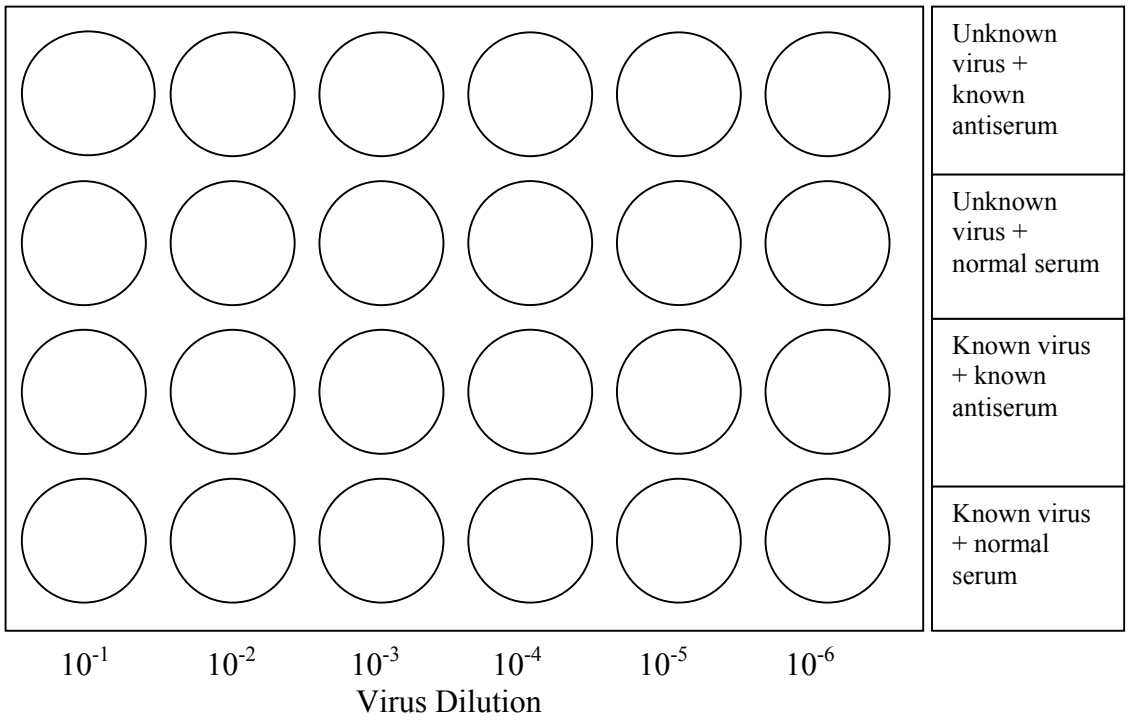
3. Combine equal volumes of each dilution of the suspect sample with the diluted antiserum. Repeat the procedure for a positive control virus. Include negative controls for both the suspect and homologous virus. Incubate for one hour with agitation at the appropriate temperature.
    a. For IHNV, VHSV, and IPNV, incubate at 15°C.

    b. For SVCV incubate at 20-25°C.

4. Inoculate each of these mixtures onto the cell line in which the suspect virus was isolated as indicated in Figure 4.13 below. Incubate at the above temperature for 14 days and observe plates for cytopathic effect (CPE) (See figures 4.1 – 4.12).
    a. **Equivalent inhibition of CPE by a specific antiserum for both the suspect and homologous virus, but not for negative controls, provides confirmatory identification of the suspect virus.**

    b. Alternatively, in a sample mixed with antibody, a titer decrease of 2 $\log_{10}$ indicates neutralization and confirms the identity of the virus.

Figure 1- Diagram for Serum Neutralization Assay using a 24 well plate



C. Preparation of Reference Viruses - Where appropriate, positive controls are produced and frozen for use as needed in the serum neutralization assay.

1. Thawing of Frozen Viral Isolates - Thaw virus isolates rapidly in lukewarm water, removing the vial just before the last of the ice in the vial has melted.

2. Procedure for Producing Reference Viruses:
    a. Inoculate viral suspensions onto cell culture flasks containing visibly healthy monolayers of the appropriate cell line. This is done in a manner similar to the tissue inoculation described above (4.5.A) using an appropriate volume of inoculum for the flask size. If using a 25cm^2 flask, 0.1 ml of viral inoculum is usually sufficient.
        i. Use EPC cell line for IHNV, SVCV, and VHSV isolates

        ii. Use CHSE-214 cell line for IPNV isolates

    b. To allow for viral adsorption, incubate flasks at the appropriate temperature for 1 hour with gentle rocking at least every 15 minutes or continuously on a laboratory rocker.
        i. For IHNV, IPNV and VHSV incubate at 15°C.

        ii. For SVCV incubate at 20-25°C.

c. Aseptically dispense an appropriate amount of tissue culture media into the flask. For a 25 cm^2 flask this will be approximately 5 ml.

d. Incubate control sample flasks to allow replication of the viruses
    i. For IHNV, IPNV, and VHSV incubate at 15°C until CPE occurs or for 14 days.

    ii. For SVCV incubate at 20-25°C until CPE occurs or for 14 days.

Procedure for Harvesting the Virus

a. Using aseptic technique, scrape the cell layer from the flask and triturate to break up.

b. Pour fluid and suspended cells into sterile tubes for centrifugation.

c. Centrifuge tubes at 4°C for 15 minutes at 2000-3000 times gravity relative centrifugal force (X g).

d. Use supernatant as positive control virus. Any fluid not needed for the assay may be aliquoted into vials and frozen at -70 C.

e. Any supernatant that is not frozen or used for the assay must be decontaminated before it is discarded.

4.8 Glossary

Blind passage - transfer of supernatant and inoculated tissue culture cells which are not demonstrating CPE to another plate containing fresh cells in order to dilute out possible inhibitors of viral expression and/or allow possible early viral replication due to low concentrations of virus particles to progress to detectable CPE.

Closed System - a system of incubating cells that is sealed against the transfer of air, i.e., a sealed flask.

Confluent Monolayer (100%) - a single layer of tissue culture cells in which the cells have filled in all the spaces between them.

Controls

A. **Monolayer control:** tissue culture cells are grown in presence of tissue culture medium. If CPE appears in monolayer control wells, test is invalidated and must be repeated.

B. **Sham control:** diluent (MEM-0) used for suspension of samples or dilution blanks is added to cells. After adsorption, tissue culture medium is added. If CPE appears in sham control wells, test is invalidated and must be repeated.

Cytopathic Effects (CPE) - changes in the morphology and metabolism of tissue culture cells. It may be due to viral or toxic agents and the appearance may range from simple foaming of the cytoplasm or focal clumping of cells to complete destruction of the cell monolayer.

FBS - fetal bovine serum taken from unborn calves in utero.

Fomite – an inanimate object such as a net, brush, or clothing, on which a pathogenic microorganism may be transmitted from one animal to another.

Homologous virus – as used in the viral serum neutralization procedure, it is the positive control virus of the same identity used to make the neutralizing antibody.

Monoclonal Antibody (MAb) - antibody produced by tissue culture cell lines derived from the spleen lymphocytes of immunized mice that have been fused (hybridoma) with mouse myeloma tumor cells. Hybridoma cells are cloned to select specific populations of cells, each producing a single antibody against one epitope or antigenic determinant site on one antigen molecule among those used to immunize the mice.

Normal Serum – as used in the viral serum neutralization procedure, it is serum from the same species of animal in which the neutralizing antibody is produced. It is used as a control for any nonspecific viral inhibition that may occur even with a non-homologous virus.

Open System - a system of incubating tissue culture cells that is open to the transfer of air, i.e., a plate. Requires a medium that is buffered against rising pH due to CO_2 loss. Common buffering systems are TRIS and HEPES.

Plaque - a hole or focus of degenerate or dead tissue culture cells in the cell monolayer caused by viral replication. One discrete plaque is assumed to be caused by infection with one infectious particle or aggregate (called one plaque-forming unit = pfu).

Plate set - a group of plates seeded from a single flask at the same time.

Polyclonal Antibody - the entire population of antibodies produced in the sera of immunized animals that are directed against many epitopes on many of the antigenic molecules used for immunization. Most immunogens injected are whole cells or viruses that are composed of many different antigen molecules. Each antigen molecule may have more than one epitope. See "Monoclonal Antibody.

Re-inoculation - transfer of inoculated tissue culture cells and supernatant from one plate to another that contains fresh cells. Used for suspected positive cultures to confirm presence of viral CPE as opposed to toxicity or contamination. Also used to replicate more viruses for storage, etc.

Serum neutralization - antibody molecules in the antiserum neutralize or block the antigenic receptor sites or otherwise degrade the protein coat (capsid) on the corresponding virus (antigen). This prevents virus attachment to and subsequent penetration of host tissue culture cells or virus replication once inside the cell. Neutralization of viruses by antibodies is specific and used to confirm viral identity. Neutralization may be reversible.

Subculture – transfer of tissue culture cells from one container to another for the purpose of forming a new monolayer.

$TCID_{50}$ - denotes fifty percent tissue culture infective dose. This is the reciprocal of the highest dilution of virus that causes CPE in 50% of the wells inoculated with that dilution of infectious materials. This is determined by the Reed and Muench (1938) method.

Tissue Culture-Grade Water - High quality water (low in ions, minerals and contaminants) that must be used in preparation of all tissue culture media and reagents and in rinsing glassware to avoid toxicity to the cells. De-ionization at greater than 17 Ohms is sufficient to achieve this quality.

Titer - the number of infectious units or plaque-forming units per unit of sample, i.e., per gram or ml.

Toxicity - changes in cell morphology or metabolism caused by toxic substances in the medium or inoculum. This can either cause cell death or interfere with cell metabolism, thereby reducing or preventing replication of the virus. These effects may have arisen through sample toxicity, bacterial or fungal contamination, improper glassware cleaning or improper

media preparation. Usually toxicity can be distinguished from viral CPE by how rapidly it occurs (1 day), abnormal cell appearance without cell death, absence of the typical pattern of CPE for the test virus, and, in the case of contamination, turbidity of the medium or visible contaminant colonies.

> **NOTE:** Inoculation of very high-titer suspensions of certain viruses can cause an apparent toxic effect within 24 hours. If there is any doubt to whether disruption of the cell layer was caused by toxicity or CPE, a subculture should be made. This is especially true for some inocula that can produce toxic effects that may take 5-7 days for development.

Triturating - The act of dispersing tissue culture cells for transfer by repeatedly drawing the cell suspension into a pipet and expelling it back into the flask. This should be done until the cells are in clumps of no more than three when examined with an inverted light microscope.

Trypsin - a proteolytic enzyme used to disperse cells and causes their release from the culture surface. Serum proteins neutralize it and its action is slowed by low temperature. Trypsin will cause release of the cells more readily than versene.

Versene (EDTA) - ethylene di-amine tetra-acetic acid is a chelating agent involved in causing cells to release from the culture surface.

4.9 Reagents and Media

A. Sample dilution medium - Hanks Balanced Salt Solution (HBSS)

10X HBSS	100.0 ml
Tissue Culture Grade Water	895.3 ml
$NaHCO_3$ (7.5%)	4.7 ml

Mix. Filter with 0.2 um filter.

B. Antibiotic incubation medium (anti-inc) made with HBSS for sample disinfection

10X HBSS	100.0 ml
Tissue Culture Grade Water	575.0 ml
$NaHCO_3$ (7.5%)	5.0 ml
Penicillin/Streptomycin	160.0 ml
Penicillin G (10,000 units/ml)	
Streptomycin sulfate (10,000 ug/ml)	
Fungizone	160.0 ml
250 ug/ml Amphotericin B	
205 ug/ml desoxycholate	

Mix. Filter with 0.2 um filter. Store at 4° C.

C. Antibiotic incubation medium (anti-inc) made with Minimum Essential Medium (MEM-0) for sample disinfection

10X MEM (Eagles Modified Medium)	100.0 ml
Tissue Culture Grade Water	540.0 ml
L-Glutamine (200 mM)	10.0 ml
$NaHCO_3$ (7.5%)	30.0 ml
Tryptose Phosphate Broth	100.0 ml
Penicillin/Streptomycin	160.0 ml
Penicillin G (10,000 units/ml)	
Streptomycin sulfate (10,000 ug/ml)	
Fungizone	160.0 ml
250 ug/ml Amphotericin B	
205 ug/ml desoxycholate	

Mix aseptically. Filter with 0.2 um filter. Can store frozen for approximately 3 months. Avoid freeze-thaw cycles, thaw tubes immediately prior to use.

D. Versene (EDTA) (1:5000)

NaCl	8.0 g
$KHPO_4$	0.2 g
KCl	0.2 g
Na_2HPO_4	1.15 g
Disodium Versenate (EDTA)	0.2 g
Phenol Red (0.5% solution)	2.0 ml
Tissue Culture Grade Water	to 1000 ml

Autoclave and store at room temp.

E. Trypsin-Versene (EDTA)

Trypsin (2.5% solution)	20 ml
Versene (EDTA) (1:5000)	480 ml

Store at -20° C.

F. MEM-5/Hepes (tissue culture medium for all cell lines except SHK-1)

10X MEM	100.0 ml
Tissue Culture Grade Water	810.0 ml
Fetal Bovine Serum	50.0 ml
Sodium Bicarbonate (7.5% solution)	10.0 ml
L-Glutamine (200 mM)	10.0 ml
Hepes Buffer (1M)	15.0 ml
NaOH (1M)	5.0 ml
NaOH or HCL	as needed to adjust pH to 7.2-7.6

If antimicrobials are included, use 796.0 ml of water above instead of 810.0 and add

Gentamicin (50 mg/ml)	4.0 ml
Fungizone	10.0 ml
250 ug/ml Amphotericin B	
205 ug/ml desoxycholate	

Mix aseptically, filter with 0.2 um filter and store at 4° C.

G. MEM-10/Hepes (tissue culture medium for all cell lines except SHK-1)

10X MEM	100.0 ml
Tissue Culture Grade Water	760.0 ml
Fetal Bovine Serum	100.0 ml
Sodium Bicarbonate (7.5% solution)	10.0 ml
L-Glutamine (200 mM)	10.0 ml
Hepes Buffer (1M)	15.0 ml
NaOH (1M)	5.0 ml
NaOH or HCL	as needed to adjust pH to 7.2-7.6

Mix aseptically, filter with 0.2 um filter and store at 4° C.

H. Leibovitz's L-15 (tissue culture medium for SHK-1 cell line)

1X L-15 with 0.3g/L L-glutamine	1000.0 ml
Fetal bovine serum (5%)	50.0 ml
Gentamicin (50 mg/ml)	1.0 ml
2-mercaptoethanol (0.055 M)	0.7 ml
NaOH or HCL	as needed to adjust pH to 7.2-7.6

Mix aseptically, filter with 0.2 um filter and store at 4° C.

4.10 References

Amos, K. H., editor. 1985. Procedures for the detection and identification of certain fish pathogens, 3rd edition. Fish Health Section, American Fisheries Society, Corvallis, Oregon.

Arakawa, CK, RE Deering, KH Higman, KH Oshima, PJ O'Hara, and JR Winton. 1990. Polymerase chain reaction (PCR) amplification of a nucleoprotein gene sequence of infectious hematopoietic necrosis virus. Dis. Aquat. Org. 8:165-170.

Batts, W. N., and R. J. Winton. 1989. Enhanced Detection of Infectious Hematopeietic Necrosis Virus and Other Fish Viruses by Pretreatment of Cell Monolayers with Polyethylene Glycol. Journal of Aquatic Animal Health. 1:284-290.

Bouchard, D., W. Keleher, H. M. Opitz, S. Blake, K.C. Edwards, and B.L. Nicholson. 1999. Isolation of Infectious Salmon Anemia Virus (ISAV) from Atlantic Salmon in New Brunswick, Canada. Dis. Aquat. Org. 35:131-137.

Bruno, D. W., T. T. Poppe. 1996. A Colour Atlas of Salmonid Diseases. Academic Press, California.

Egusa, S. 1991. Infectious Diseases of Fish. Oxonian Press PVT. Ltd., New Delhi.

Einer-Jensen K, NJ Olesen, N Lorenzen, PEV Jorgensen. 1995. Use of the polymerase chain reaction (PCR) to differentiate serologically similar viral hemorrhagic septicemia (VHS) virus isolates from Europe and America. Vet Res 26: 464-469.

Freshney, R. I. 1983. Culture of Animal Cells: A Manual of Basic Technique. Alan R. Liss, Inc., New York.

Jakoby, W. B. and I. H. Pastan. 1979. Methods in Enzymology Volume LVIII: Cell Culture. Academic Press, Inc., California.

Merchant, D. J., R. H. Kahn, and W. H. Murphy. 1964. Handbook of Cell and Organ Culture. 2nd Edition. Burgess Publishing Company, Minnesota.

OIE Diagnostic Manual for Aquatic Animal Diseases. 2000. 3rd Edition. Office International Des Epizooties. Paris France.

Olesen, N. J., Jørgensen, P. E. V. (1992) Comparative susceptibility of three fish cell lines to Egtved virus, the virus of viral haemorrhagic septicaemia (VHS). Dis. Aquat. Org. 12: 235-237.

Plumb, J. A., 1999a. Health Maintenance and Principal Microbial Diseases of Cultured Fishes. Iowa State University Press, Ames, IA 50014.

Plumb, J. A., A.D Noyes, S. Graziano, J. Wang, J. Mao, and V. G. Chinchar. 1999b. Isolation

and identification of viruses from adult largemouth bass during 1997-1998 survey in the southeastern United States. Journal of Aquatic Animal Health 11:391-399.

Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. American Journal of Hygiene 27:493-497

Roberts, R. J., 1982. Microbial Diseases of Fish. Academic Press, New York.

Rovozzo, G. C. and C. N. Burke. 1973. A Manual of Basic Virological Techniques, Prentice-Hall, New Jersey.

Thoesen, J. C., (ed.). 1994. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens (4th Edition) Fish Health Section, American Fisheries Society, Bethesda, MD.

True, K., (ed.). 2000. National Wild Fish Health Survey: Laboratory Procedures Manual, First Edition. United States Fish and Wildlife Service.

Wolf, K., 1988. Fish viruses and fish viral diseases. Cornell University Press, New York.

4.A1 PCR Worksheets

A. Worksheet A - PCR Sample Data/Log Sheet

PCR Sample Data/Log Sheet

Case Number_____ **Sample Site**_____ **Date**_____
Species_____
Tissue type_____

Sample ID	Extraction Method	PCR Result	Notes
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			
12.			
13.			
14.			
15.			
16.			
17.			
18.			
19.			
20.			

B. Worksheet B - Amplification of Nucleic Acid by PCR for the Confirmation of Viral Fish Pathogens

1. Infectious Hematopoietic Necrosis Virus (IHNV)

2. Infectious Salmon Anemia Virus (ISAV)

3. Largemouth Bass Virus (LMBV)

4. Viral Hemorrhagic Septicemia Virus (VHSV)

Worksheet 4.A1.B.1: Infectious Hematopoietic Necrosis Virus (IHNV)

Case Number_____ Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	23.75 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		2.5 mM	25 mM	5 µl	
dNTP's		0.2 mM	2 mM	5 µl	
AMV Reverse Transcriptase		4.5 U/Rx	9 U/µl	0.5 µl	
(+)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
(-)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
RNasin		9.75 Units/Rx	39 Units/µl	0.25 µl	
RNA Template		-	-	5 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Master Mix for Nested or Second Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	27.5 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		2.5 mM	25 mM	5 µl	
dNTP's		0.2 mM	2 mM	5 µl	
(+)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
(-)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
Round 1 Product		-	-	2 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for IHNV

	Forward	**Reverse**
1st round	5'-TCA AGG GGG GAG TCC TCG A-3'	5'-CAC CGT ACT TTG CTG CTA C-3'
2nd round	5'-TTC GCA GAT CCC AAC AAC AA-3'	5'-GCG CAC AGT GCC TTG GCT-3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				
2nd round				

Amplification (Thermocycle Process)

Round Number (Date & time)	**Program #**	**NOTES**

Gel Preparation

Gel Concentration	**Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)**	**Weight of agarose (grams)**	**Volume of Buffer (ml)**

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

Worksheet 4.A1.B.2: Infectious Salmon Anemia Virus (ISAV)

Case Number_____ Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	26.75 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		2.5 mM	25 mM	5 µl	
dNTP's		0.2 mM	2 mM	5 µl	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µl	0.5 µl	
(+)Primer		50 pmoles/Rx	50 pmoles/µl	1 µl	
(-)Primer		50 pmoles/Rx	50 pmoles/µl	1 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
RNasin		9.75 Units/Rx	39 Units/µl	0.25 µl	
RNA Template		-	-	5 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for ISAV

	Forward	**Reverse**
1st round	5'-GGC TAT CTA CCA TGA ACG AAT C-3'	5'-TAG GGG CAT ACA TCT GCA TC-3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	**Program #**	**NOTES**

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of agarose (grams)	Volume of Buffer (ml)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

Worksheet 4.A1.B.3: Largemouth Bass Virus (LMBV)

Case Number_____ Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µl) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	15.5 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		1.5 mM	25 mM	3 µl	
dNTP's		0.8 mM	10 mM	4 µl	
TMAC		40 µM	100 µM	20 µl	
(+)Primer		50 pmoles/µl	100 pmole/µl	0.5 µl	
(-)Primer		50 pmoles/µl	100 pmole/µl	0.5 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
DNA Template		-	-	1 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for LMBV

	Forward	**Reverse**
1st round	5'-GAC TTG GCC ACT TAT GAC-3'	5'-GTC TCT GGA GAA GAA GAA-3'

Control Information

	POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
	Extraction	PCR	Extraction	PCR
1st round				

Amplification (Thermocycle Process)

Round Number (Date & time)	**Program #**	**NOTES**

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of agarose (grams)	Volume of Buffer (ml)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

Worksheet 4.A1.B.4: Viral Hemorrhagic Septicemia Virus (VHSV)

Case Number_____ Date_____

Master Mix for Initial or First Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	23.75 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		2.5 mM	25 mM	5 µl	
dNTP's		0.2 mM	2 mM	5 µl	
AMV Reverse Transcriptase		4.5 Units/Rx	9 Units/µl	0.5 µl	
(+)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
(-)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
RNasin		9.75 Units/Rx	39 Units/µl	0.25 µl	
RNA Template		-	-	5 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Master Mix for Nested or Second Round Reaction

PCR Reagent	Lot #	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		-	-	27.5 µl	
PCR Buffer (no $MgCl_2$)		1X	10X	5 µl	
$MgCl_2$		2.5 mM	25 mM	5 µl	
dNTP's		0.2 mM	2 mM	5 µl	
(+)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
(-)Primer		50 pmoles/Rx	20 pmoles/µl	2.5 µl	
TAQ		2.5 Units/Rx	5 Units/µl	0.5 µl	
Round 1 Product		-	-	2 µl	-

*Add nuclease free water to Master Mix first, TAQ last.

Primer Sets for VHSV

	Forward	Reverse
1st round	5'-TCT CTC CTA TGT ACT CCA AG-3'	5'-TTC CGG TGG AGC TCC TGA AG-3'
2nd round	5'-ATG GGC TTC AAG GTG ACA C-3'	5'-GTA TCG CTC TTG GAT GGA C-3'

Control Information

	POSITIVE CONTROLS		NEGATIVE CONTROLS	
	Extraction	PCR	Extraction	PCR
1st round				
2nd round				

Amplification (Thermocycle Process)

Round Number (Date & time)	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of agarose (grams)	Volume of Buffer (ml)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

C. Worksheet 4.A1.C - Photodocumentation of the PCR Product Gel

Case Number _____ Date:

Samples

Affix gel photo documentation to this sheet and make notes on results:

AFFIX PHOTODOCUMENTATION

Notes:

4.A2 Cell Enumeration (True, 2000)

Rarely are cells counted during routine propagation of cell lines, however the use of a hemocytometer is a practical method for determining cell numbers in cell suspensions. The Improved Neubauer Hemocytometer consists of two chambers, each of which is divided into nine 1.0-mm^2 squares. A matching cover glass that is supplied with the chamber is supported 0.1 mm over the squares so that the total volume over each square is 1.0 mm^2 x 0.1 mm or 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is approximately equal to 1 ml, the cell concentration/ml is the average count per square x 10^4. Routinely, cells are counted in a total of ten 1 mm squares (fill both sides of the chamber and count the four corner and the middle squares on each side).

To reduce counting errors, count cells that touch the outer line to the left and top of the square, but do not count cells touching the outer line to the right and bottom of the square. Hemocytometer counts do not distinguish between living and dead cells unless a vital stain is used such as Trypan Blue.

Trypan Blue stain is not absorbed by living cells and can be used to distinguish between viable and nonviable cells in cell counts. Use a 1:1 dilution of cell suspension with 0.1% Trypan Blue stain and count only unstained cells. Do not count debris or dead cells that stain blue.

Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted (10 min at 500 rpm) and re-suspended in protein-free medium or Hanks salt solution prior to counting.

A. MATERIALS

Hemocytometer chamber
75 cm^2 flask of cells
Trypan Blue (0.1% in PBS)
Microscope
Dilution tubes (12 x 75 mm)
Pasteur pipet
Hanks balanced salt solution, or MEM-0 (MEM w/o serum)
Trypsin - EDTA
Pipets 1-ml, sterile, cotton plugged
22 x 22 mm cover-slips

B. PROCEDURE

1. Select a healthy (log phase) 75 cm^2 flask of cells and remove cells from flask surface as described in 4.3A.

2. Re-suspend cells in tissue culture medium (MEM-0). For ease and accuracy in counting, the hemocytometer should be filled with cell suspensions containing approximately 20-50 cells/mm^2 (1 x 10^5 to 2 x 10^5 cells/ml). Dilutions vary depending on age of the cells, cell density and cell aggregation.
3. Aseptically transfer 0.5 ml of the cell suspension into a dilution tube.

Add 0.5 ml Trypan Blue stain (0.1%).

> **Note:** If cells are exposed to Trypan Blue for extended periods of time, viable cells, as well as non-viable cells, may begin to take up dye.

4. Gently mix to suspend the cells evenly. With a 22 x 22 mm cover-slip in place on top of the hemocytometer, use a Pasteur pipet to transfer a small drop of Trypan Blue-cell suspension mixture to both chambers. Carefully touch the edge of the cover-slip with the pipet tip and allow each chamber to fill by capillary action. Don't overfill or underfill the chambers.

5. Using a microscope with a 10x ocular and a 10x objective count 10 squares (5 from each chamber) as outlined above.

6. Calculate the # of cells/ml and the total # of cells as follows:

   Cells/ml = x (mean) count per square x 10^4 x Trypan Blue dilution factor

   Total cells in flask = cells/ml x total volume of cell suspension

   e.g., total # cells counted in 10 squares = 300 cells

   x count/square = 300 cells/10 squares = 30 cells

   cells/ml = 30 x 10^4 x 2 (dilution factor)
   cells/ml = 60 x 10^4 cells/ml
   cells/ml = 6.0 x 10^5 cells/ml

   Total cells = 6.0 10^5 cells/ml x 8 ml (original volume cell suspension)
   Total cells = 48.0 x 10^5 cells
   Total cells = 4.80 x 10^6 cells

If the cells/ml calculated is not within the recommended range of cell density, use the following formula to adjust the dilution in your flask before splitting.

ml medium needed = (actual cells/ml) (vol. of cell suspension)/ desired cells/ml

e.g., actual count = 6 x 10^6 cells/ml
desired count = 1 x 10^6 cells/ml
volume of cell suspension = 8 ml
ml medium needed = x

x = ml medium needed = 6 x 10^6 cells/ml x 8 ml / 1 x 10^6 cells/ml

$$\text{ml medium needed} = 48 \times 10^6 \text{ ml} / 1 \times 10^6$$

$$= 48 \text{ ml}$$

Since you have 8 ml already in the flask, you would need to add 40 ml of medium to the flask before splitting to get the recommended seeding cell density for each new culture.

Chapter 5

Parasitology

5.1 Introduction

The following chapter describes identification procedures for four parasitic infections of fish that are commonly included in a fish health inspection. The target parasite species include three myxozoan parasites of salmonid fishes – *Myxobolus cerebralis, Ceratomyxa shasta and Tetracapsula bryosalmonae* - and the cestode *Bothriocephalus acheilognathi,* which infects members of the Family Cyprinidae. Chapter 2 describes procedures for proper sampling of fish tissues to ensure detection of any of these pathogens during a fish health inspection.

For *Myxobolus cerebralis*, presumptive identification is based on identification of the myxozoan spore stage from pepsin-trypsin digested (PTD) cartilage. Tissues from up to 5 fish may be pooled for screening by PTD. Identification of the spores is based on morphology. Confirmatory identification is based on identification of the spores in histological sections, or on amplification of *M. cerebralis* DNA by the polymerase chain reaction.

For *Ceratomyxa shasta*, presumptive identification is based on identification of myxozoan spore or trophozoite/presporogonic stages from intestinal tissue. Identification of the myxospore stage is sufficient for confirmation of infection. Identification of the earlier stages must be confirmed by amplification of *C. shasta* DNA by the polymerase chain reaction.

For *Tetracapsula bryosalmonae*, presumptive identification is based on identification of the presporogonic stages of the parasite in Leishman-Giemsa or lectin stained imprints of kidney or spleen tissue. Infection is confirmed by identification of these stages in histological sections of kidney tissue.

For the cestode, *Bothriocephalus acheilognathi,* visualization of any cestode with a pyramidal scolex results in a presumptive positive classification. Confirmation requires verification of morphological characteristics of the scolex.

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U.S. Fish and Wildlife Service, the United States government and /or the American Fisheries Society. Any comparable instrument, laboratory supply or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

5.2 *Myxobolus cerebralis* (Whirling Disease)

Myxobolus cerebralis may be difficult to detect because the life cycle of the parasite includes two alternate hosts (salmonids and the aquatic oligochaete worm, *Tubifex tubifex*) (Wolf and Markiw 1984) and the extended time required for sporogenesis in the salmonid host. For an *M. cerebralis* inspection, sampling should target the most appropriate species, age, and rearing units that are most likely to reflect the presence of the parasite. The species most susceptible are rainbow trout, sockeye salmon, and steelhead. Brook trout, cutthroat trout, Atlantic salmon and chinook salmon are moderately susceptible; brown and bull trout, coho salmon and splake are partially resistant. Conflicting data are present for Arctic grayling and lake trout but in general these species may be considered resistant or partially resistant to *M. cerebralis*. Fish are most susceptible if exposed when young, however, older fish may become infected and act as carriers of spores. Fish that have a high degree of resistance should not be selected for sampling unless they are the only species present. (O'Grodnick 1979; Hedrick et al. 1999a, b; Hedrick et al. 2001; MacConnell and Vincent 2002).

Development of myxospores is temperature dependent, requiring a minimum of 90 d at 16-17˚C, and 120 d at 12-13˚C (El-Matbouli et al. 1992). When temperature data is available, sample fish that have been on the water supply for a minimum of 1800 degree-days. If continuous temperature monitoring data is not available, select fish that have been on the water supply for a minimum of 6 months. Select fish that reside in locations on an aquaculture facility most likely to result in exposure to the parasite such as earthen rearing containers and from locations receiving untreated surface water.

Screening for *M. cerebralis* is by examination for spores in cranial cartilage processed by pepsin-trypsin digest. Up to 5 fish may be pooled for screening; confirmation is on individual fish. Confirmation of *M. cerebralis* is by identification of parasite stages in histological sections of cartilage tissue, or by amplification of parasite DNA by the polymerase chain reaction (PCR).

A. Screening Test

Pepsin-trypsin Digestion (PTD) (Markiw and Wolf 1974; Lorz and Amandi 1994). This method is recommended for fresh samples; if the sample has been frozen, follow the procedure below with modifications noted in A.3a and A.4.

1. Defleshing
    a. Samples should be placed in an appropriate container and heated at 45 °C in a water bath until flesh is soft and eyes are opaque.

    b. Deflesh the samples and retain all cartilage/bone including that from the gill arches and opercula.

Pepsin Digestion

a. Weigh cartilage and add 0.5% pepsin solution (5.6.A) at a ratio of 20 ml/g cartilage.

b. Large heads (greater than 20 g after defleshing) may be homogenized in the pepsin solution with an electric blender.

c. Stir at 37 °C and monitor pH of pepsin solution. If pH increases above 4.0, centrifuge samples at 1200 x g for 10 minutes, decant pepsin and add fresh pepsin to the sample.

d. Process samples until all cartilage/bone is reduced to a consistently small granular size (e.g. the size of beach sand; 1 h generally sufficient for small fish; 2 h to overnight may be required for larger fish).

e. When digestion is complete, centrifuge at 1200 x g and decant pepsin into a solution of 1:4 bleach:water (5,000 ppm chlorine) for disposal.

Trypsin Digestion

a. Add 0.5% trypsin (5.6.B) at a ratio of 20:1 (volume/g) to the pellet.

> **Note**: If samples have been frozen prior to processing, reduce the concentration of trypsin to 0.05%

b. Adjust to pH 8.5 with 1 N sodium hydroxide (NaOH; 5.6.C).

c. Stir at room temperature for 30 minutes.

d. If samples are to be refrigerated at this stage, inactivate trypsin by adding serum to a final concentration of 20%, or powdered bovine serum albumin to a final concentration of 1%.

e. Pour digested sample through a non-absorbent disposable filter [e.g. paint filters (any paint store), urinary calculi filters, or synthetic material such as nylon screen] into a new tube. Mesh sizes should approximate 200 µm. Reusable filters are not recommended because of difficulties in decontamination. Autoclave filters before disposal.

f. Centrifuge at 1200 x g for 10 minutes.

g. Discard supernatant into a solution of 1:4 bleach:water (5,000 ppm chlorine) while retaining pelleted material.

Dextrose Centrifugation (Markiw and Wolf 1974). This concentration step is recommended if spores are not found on initial examination or if a large amount of tissue debris makes microscopic examination difficult; it is not recommended if samples have been frozen because it causes distortion and decreased recovery of the spores

a. Add 1 ml of phosphate buffered saline (PBS) (5.6.D) to the sample and vortex (this volume may need to be adjusted depending on pellet size, but should be sufficient to suspended all material).

b. A 55% solution of dextrose is used to provide a 5 cm deep gradient in a 15 ml centrifuge tube. For larger samples, a 50 ml centrifuge tube may be required.

c. Carefully layer the suspended sample onto the dextrose solution.

d. Centrifuge at 1200 x g for 30 minutes.

e. Carefully decant the supernatant into a solution of 1:4 bleach:water (5,000 ppm chlorine) while retaining all pelleted material.

f. Add 0.5 – 1.0 ml of neutral buffered formalin (5.6.J) to the pellet (a 10 fold dilution of the pellet should be sufficient to disperse material enough to see clearly under the microscope). Fixed samples can be stored at room temperature until examined.

5. Analyzing Samples
   a. Vortex samples prior to preparing slide.

   b. Samples may be examined stained or unstained by wet mount or using a hemocytometer. A number of simple staining methods are suitable for staining spores; e.g. add 60 µL of a saturated solution of crystal violet biological stain to 1 ml subsample, mix and examine.

   c. A minimum of 150 fields should be examined at 200x magnification (20x objective with 10x ocular eyepiece). Measure ten representative myxospores and record the average size and size range. Identification of myxospores with the appropriate size and morphology (8 – 10 µm, rounded, two polar capsules; Figures 5.1) results in a sample designation of **PRESUMPTIVE positive.** If the size is appropriate but internal morphology is not clear, the sample should also be identified as **PRESUMPTIVE positive.** A single myxospore of appropriate size is sufficient to declare a **PRESUMPTIVE positive**. **All other samples are reported as negative.**

      Note: Other myxobolid species of salmon have similar morphologies that may be confused with *M. cerebralis*. Photographs and measurements of some of these species are provided in Figure 5.2 for comparison.

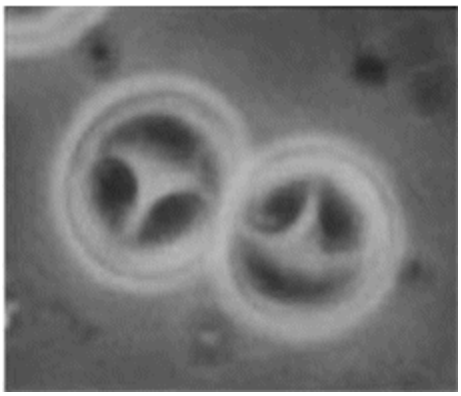
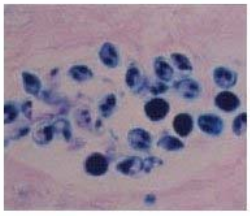


Figure 5.1. Spores of *Myxobolus cerebralis* Photograph courtesy of R. P. Hedrick

Figure 5.2: Myxozoans Common To Salmonid Fish

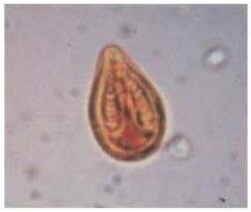
Myxobolus cerebralis

L 7.4 - 9.7 x W 7 - 10 x T 6.2 x 7.4 μm



Myxobolus kisutchi

L 8.5 x W 7 μm



Myxobolus insidiosus

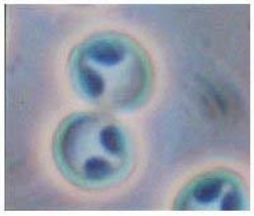
L 12.8 - 17.1 x W 8 - 11.9 μm

Myxobolus arcticus

L 14.3 - 16.5 μm

Myxobolus neurobius

L 10 - 14 x W 8 - 9.2 μm

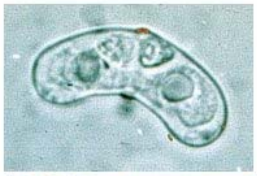


Myxobolus squamalis

L 8.4 - 9.9 x W 7.7 - 9.9 μm

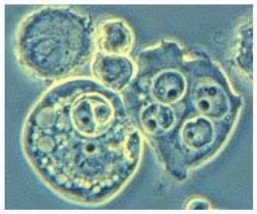
Myxidium minteri

L 9.3 - 12.6 x W 4.5 - 5.3 μm

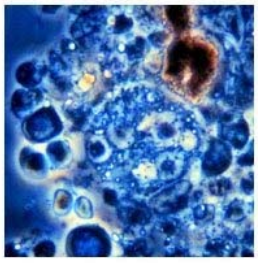


Ceratomyxa shasta

L 14 - 23 x W 6 - 8 μm



Ceratomyxa shasta trophozoites



PKX organism

B. Confirmatory Tests

Confirmation of *M. cerebralis* should rely on either histopathology or the nested version of the polymerase chain reaction (PCR).

1. Histopathology
    a. Half heads, wedges or core samples, which were placed in 10% formalin or Davidson's fixative at the time of collection (2.2.E.4) will be used for corroboration. Process tissues corresponding to a sample number in which a presumptive positive was found.

    b. Small samples (from fish <15 cm) fixed in Davidson's can be transferred to 70% ethanol after 24-48 h. Larger samples should remain in Davidson's for 48 h and may require an additional decalcification step (as specified below).

    c. Samples fixed in 10% formalin should be transferred after 24-48 h to a commercially available chemical decalcification solution (e.g., Lerner-D-Calcifier, Decal-Stat Solution, Cal-Ex and others) or acid decalcifying solution (e.g., 10% Nitric Acid, 5% Formic Acid).

    d. Samples requiring decalcification are placed into embedding cassettes in a beaker with a magnetic stir bar and sufficient decalcifying solution (20:1 solution to gram of head tissue). Covered and place on a stirring plate for 4-16 h at room temperature. After 3 h, and periodically thereafter, use physical and/or chemical tests to determine if the process is complete (Hauck and Landin 1997):
        i. Physical. Probe using a dissecting needle to determine softness of bone and/or cartilage (avoid puncturing the tissues). Soft tissues are adequately decalcified.

        ii. Chemical. Remove 5 ml of decalcifying solution from beaker and neutralize (pH 7.0) with 0.1 N sodium hydroxide (5.6.C stock soln). Add 1 ml of saturated ammonium oxalate solution and mix. Allow to stand for 15 min and determine if a precipitate (slight cloudiness) of calcium oxalate forms. If so, the decalcification is incomplete. If incomplete, replace decalcifying solution and continue decalcification process until no calcium oxalate precipitate can be detected.

        iii. After decalcification, rinse specimens in distilled water and transfer via graded ethanol series to 70% ethanol.

    e. Embed and section tissues at approximately 4-5 µm using standard methods. Two sections, taken at 100 µm apart, should be mounted from each sample (or each tissue block if more than 1 block per fish) and stained with Giemsa (May-Grunwald Giemsa works well, Luna 1968) or Hematoxylin and Eosin (H&E). The sections should target gill arches (especially from brown trout) and the ventral calverium (floor of the brain case). Examine slide at 200X for cartilage lesions, spores and developing stages of *M. cerebralis* (Figure 5.3).

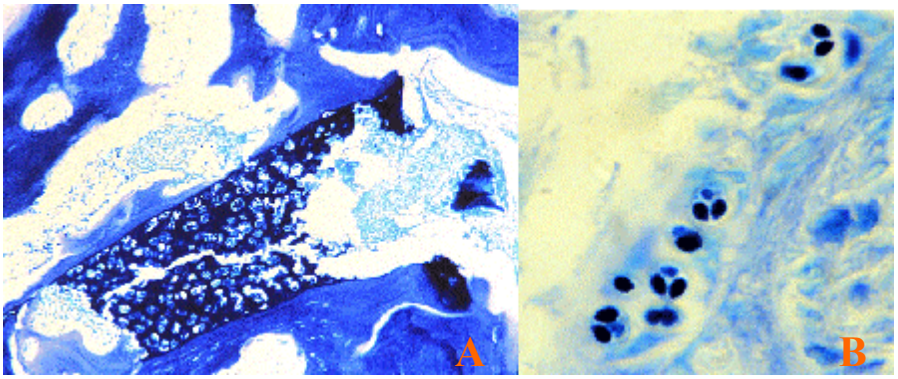


Figure 5.3. Histological sections from a fish infected with *Myxobolus cerebralis*. A.) Low magnification showing degradation of cartilage (Leishman-Giemsa stain). B.) High magnification showing stained spores (note darkly stained polar capsules). Photograph courtesy of H. V. Lorz.

f. At least two additional sections (100 µm apart) should be taken, mounted and stained if histological lesions and/or spores are not observed.

If histopathology has not resulted in the identification of any stages of *M. cerebralis*, the results are reported as negative. Observation of any presporogonic or sporogonic stage of *M. cerebralis* is reported as positive.

2. Polymerase Chain Reaction (PCR) (Andree et al. 1998; Schisler et. al., 2001) - PCR should be utilized as a confirmatory test *only* on tissue from half-heads or core samples collected as described under specimen collection for PCR at the time of inspection.
   a. General Protocols and QA/QC Considerations (see Chapter 6 for Specific QA/QC considerations for PCR):

   b. Extraction of DNA –

      Note: The following protocol recommends use of the Qiagen DNeasy Tissue Kit (Qiagen Inc, Valencia, CA; Qiagen #69506). Although other kits may work similarly, they have not been tested for this application. Use the kit as per the handbook, with the following modifications:

      i. Place half-head or core sample in screw capped tubes appropriate for the sample size. Add tap water to cover the tissue and heat in a water bath at 95 °C for 15 minute. For heads ≥ 5 centimeters (2 inches), use 8 ounce sample cup or small beaker and heat for 20 minutes.

ii. Pour water into a solution of 1:4 bleach:water (5,000 ppm chlorine) for 30 min to disinfect and place fish sample on polypropylene cutting mat or other surface that can be disinfected or disposed of (autoclave).

iii. Deflesh head using a clean scalpel and forceps; place bone and cartilage in a 1.7 ml micro centrifuge tube, or a 50 ml polypropylene screw cap centrifuge tube for larger samples.

iv. Add a sufficient volume of ATL buffer, such that it is mixed with the bone material approximately 1:1 (v:w) and proteinase K solution equivalent to 1/10 volume of the buffer. If glass beads are used, extra beads can be added for the larger heads.

tissue lysis buffer	ATL buffer (Qiagen #69504)
Proteinase K solution (5.6.E)	(17.86 mg/ml)
150 mg small glass beads (optional)	710 – 1180 µm, acid washed
3 large glass beads (optional)	3 mm, acid washed

v. Vortex until the sample is broken up.

vi. Incubate at 55 °C for 60 minutes with occasional vortexing for approximately 15 – 30 seconds three to four times during incubation.

vii. Centrifuge at 16,000 x g for 5 minutes

viii. Add 200 µl *aqueous* supernatant to clean micro centrifuge tube.

Note: Occasional clear layer above the white lipid layer is oil; aqueous layer is between lipid layer and debris/glass bead pellet and may be turbid. Dark particulates do not affect DNA yield but can increase column spin times required.

ix. Add 20 µl RNase A (5.6.F)(20 mg/ml; Qiagen #19101), vortex until mixed thoroughly incubate at room temperature for 2 minutes.

x. Follow kit instructions for elution of DNA using buffers and spin column supplied in the kit (DNeasy Tissue Kit; Qiagen #69506), with the following precautions:

Note: "Dirty" preps may require longer spins. DNA preps from positive samples give consistent results when using spin columns. When expecting very small amounts of DNA, the volume of elution buffer can be reduced to 50 µl.

c. Quantitation of DNA - it is advisable that extracted products be measured using a spectrophotometer to ensure that enough DNA was successfully extracted. Quantification guidelines are in 6.2.C.4

> **Note:** Quantify DNA of a representative sample (5% or 6%) from each group of a particular size range and assume all those within that size range have a similar concentration. If they do not range too widely, average the values and determine the DNA concentration. Then add an appropriate volume to each PCR assay such that the amount per reaction is between 100 and 300 ng.

d. Amplification of *M. cerebralis* DNA

i. Following general PCR protocols (6.2) use worksheets 5.A1.A to record appropriate data for PCR and 5.A1.B.1 to calculate reagent volumes that go into the Master Mix (MM). The reagents and primers for this reaction are:

1° Master Mix: 50 µl total reaction volume

H_2O – sterile, molecular biology grade	32.1 µl
Mg Cl_2 (50 mM)(5.6.K)	1.5 µl (final concentration 1.5 mM)
10X Taq Polymerase Buffer	5 µl
dNTPs (5 mM stock)	4 µl (final concentration 0.4mM)
Tr5-16 Primer 1 (20 µM)	2 µl (final concentration 0.8 µM)
Tr3-16 Primer 2 (20 µM)	2 µl (final concentration 0.8 µM)
Taq Polymerase (5 units/µl)	0.4 µl (2 units per reaction)

Primers (Andree et al. 1998)

Tr5-16 = 5'-GCA TTG GTT TAC GCT GAT GTA GC-3'
Tr3-16 = 5' -GAA TCG CCG AAA CAA TCA TCG AGC TA-3'

ii. Add PCR reagents **except for sample DNA** to the MM tube in the order listed on Worksheet 5.A1.B.1, adding water first and Taq last. Keep all reagents cold in frozen cryo-rack during mixing, and return them to freezer immediately after use.

iii. Place 48 µl of MM into each 0.5ml PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

iv. Load 2 µl of each sample DNA to the appropriately labeled PCR tubes.

v. Thermocycler should be programmed for 35 cycles of the following regime, and recorded on Worksheet 5.A1.B.1:

Cycle Parameters:

Denature at 95 °C for 5 minutes
Then, 35 cycles at:
95 °C for 1 minute
65 °C for 2.5 minutes
72 °C for 1.5 minutes

Note: For maximum sensitivity with weak positive samples these conditions are critical.

vi. For the nested amplification prepare the 2nd MM as before, but substitute primers Tr5-17 and Tr3-17. Transfer 1µl of the amplification product from the first amplification to the appropriately labeled reaction tube containing the 2nd MM and load the thermal cycler using the same program as for the first amplification.

Primers (Andree et al. 1998)
Tr5-17 = 5' -GCC CTA TTA ACT AGT TGG TAG TAT AGA AGC-3'
Tr3-17 = 5'-GGC ACA CTA CTC CAA CAC TGA ATT TG-3'

e. Visualization of PCR product by electrophoresis

i. Prepare agarose gel as indicated in Chapter 6.3.C and load 6.0 µl of each PCR reaction + 1.5 µl into sample wells.

ii. After electrophoresis of products (6.3.D), stain and photograph gel as described in 6.3.E-G.

iii. Carefully record locations of bands on positive control samples in relation to DNA ladder bands. **Detection of the expected 508 bp (base pair) amplicon in a sample will result in corroboration of the presence of *M. cerebralis* in the sample. Any samples not yielding this band with no apparent assay problems is reported as negative.**

iv. If any unusual band are present in the samples or if bands are present in negative controls, re-run PCR reaction from template DNA tube.

v. **Photo document all gels** and attach the photo to the case history information (Worksheet 5.A1.C).

5.3 *Ceratomyxa shasta* (Ceratomyxosis)

Ceratomyxa shasta is difficult to detect because the life cycle of the parasite includes two alternate hosts (salmonids and the freshwater polychaete worm, *Manayunkia speciosa*) (Bartholomew et al. 1997). Salmonids are the only known fish hosts for *C. shasta.* Although susceptibility may vary between species and strain, all salmonids should be considered susceptible. The parasite is enzootic in a number of waters in California, Oregon, Washington, Idaho, Alaska and B.C., Canada (Bartholomew et al. 1989a), but has not been reported outside of that region. Diagnosis is complicated by the long period required for development of mature myxospores in the fish, and by the pleomorphic appearance of the presporogonic stages. Parasite development is temperature dependent, but in general, at 12 ºC, a minimum of 40 days is required for spore development in rainbow trout in the laboratory. Confirmation of *C. shasta* is based on identification of myxospores with the appropriate morphology or by PCR confirmation of presumptive presporogonic life stages.

A. Screening Test

Examination of Wet Mounts (Bartholomew et al. 1989a, Bartholomew 2001)

1. To prepare a wet mount, the intestine is removed, placed on a disposable surface and opened longitudinally with a clean scalpel or scissors. Scrape the posterior 1/3 of the intestinal mucosa and mix in a drop of water on a microscope slide. Prepare wet mounts from any areas of hemorrhage as well.

2. Scan wet mount in a systematic manner under phase contrast or bright field microscopy at 200-440X magnification. Examine the entire smear, or an area equal to that under a 22 mm^2 coverslip.

3. **Presumptive diagnosis is based on identification of multicellular myxosporidian pre-sporogonic stages (trophozoites)** (Figure 5.4A). **Visualization of prespore stages is not sufficient for definitive diagnosis. Any samples in which the organisms are not seen may be discarded and reported as negative.**

B. Confirmatory Test

Confirmation of *C. shasta* should rely on either detection of mature spores or on amplification of parasite DNA using the polymerase chain reaction (PCR).

1. **Morphology** (Bartholomew 2001): **Confirmatory diagnosis of *C. shasta* is based on identification of the characteristic myxospore** (Figure 5.4B). Myxospores of *C. shasta* measure 14 to 23 μm long and 6 to 8 μm wide at the suture line. The ends of the spores are rounded and reflected posteriorly and the suture line is distinct.

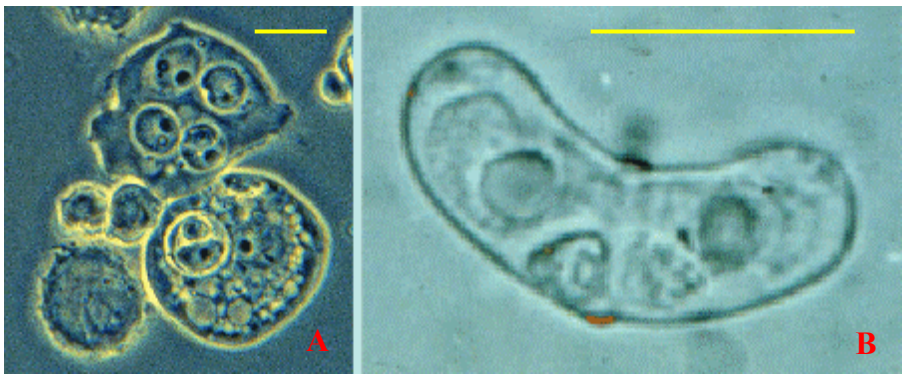


Figure 5.4. Wet mounts of: A) presporogonic stages of *Ceratomyxa shasta*. B) myxospore stage of *C. shasta***.** Bars equal 10 µm. Photograph courtesy of J. L. Bartholomew

2. **Polymerase Chain Reaction** (Palenzuela et. al. 1999; Bartholomew 2001; Palenzuela and Bartholomew 2001) - PCR should be utilized as a confirmatory test *only* on tissue collected at the time of inspection, using methods described under specimen collection.
    a. General Protocols and QA/QC Considerations (see Chapter 6 for Specific QA/QC considerations and General Protocols for PCR):

    b. Extraction of DNA:
        i. If the sample was fixed in ethanol, transfer to micro centrifuge tube with 500 µl DNA extraction buffer (5.6.G); if frozen, add 500 µl DNA extraction buffer.

        ii. Add Proteinase K solution (5.6.E) to a final concentration of 200 µg/ml (example: if stock solution is 20 mg/ml, add 5 µl).

        iii. Incubate micro centrifuge tubes at 37°C in a horizontal position on a slow platform rocker or with frequent inversion by hand. Digestion of sample will require about 4-5 h, but overnight incubation does not affect the quality of the DNA and is recommended.

        iv. After samples are completely digested, add 5 µl of RNAse A (10 mg/ml stock)(5.6.F) to each micro centrifuge tube and incubate 1 h at 37°C with rocking.

        v. Heat samples at 100°C for 5 minutes (in a water bath, heat block or thermocycler).

        vi. Remove rack and cool at room temperature. Upon cooling, samples are ready for dilution and PCR. Samples at this stage may be stored at -20°C.

c. Amplification of *C. shasta* DNA

i. Dilute the DNA template (sample) 1:10 with ultra pure (molecular grade) sterile water.

Note: Because this assay utilizes crude DNA, quantitation by UV spectrophometer does not provide useful information. A 1:10 dilution is generally sufficient unless the tissue size is larger than recommended (if this is suspected, include a 1:100 dilution in addition)

ii. Following general PCR protocols (6.3), use worksheets 5.A1.A to record appropriate data for PCR and 5.A1.B.2 to calculate reagent volumes that go into the Master Mix (MM). This assay was optimized using reagents from Promega (Madison, WI, USA) and if other buffer systems are substituted, these should be tested. The primers and reagents for this reaction are:

Master Mix: 20 µl total reaction volume

H_2O – sterile, molecular biology grade	14.6 µl
Mg CL_2 (25 mM; Promega #A351B)	1.6 µl
10X PCR Poly Buffer (Promega #M190A)	2.0 µl
dNTPs (at 10mM each)	0.4 µl (final concentration 0.2 mM)
Forward Primer (100 µM)	0.1 µl (final concentration 0.5 µM)
Reverse Primer (100 µM)	0.1 µl (final concentration 0.5 µM)
Taq Polymerase (5 units/µl)	0.2 µl (1U per reaction)

Primers (Palenzuela and Bartholomew 2001):

Forward: 5' GGGCCTTAAAACCCAGTAG 3'
Reverse: 5' CCGTTTCAGGTTAGTTACTTG 3'

iii. Place 19 µl of MM into each 0.2ml PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

iv. Add 1 µl of each sample DNA to the appropriately labeled PCR tubes.

v. Thermocycler should be programmed for 35 cycles of the following regime:

Cycle Parameters:

Denature at 95°C for 3 minutes

35 Cycles of:

94°C 1 minute

58°C 30 seconds

72°C 1 minute

72°C for 10 min.

Hold to 4°C chill at end of program.

d. Detection
    i. Prepare agarose gel as indicated in section 6.3.C and load 10.0 µl of each PCR reaction + 1.5 µl loading buffer (5.6.H) into sample wells.

    ii. After electrophoresis of products (6.3.D), stain and photograph gel as described in 6.3.E-G.

    iii. Carefully record locations of bands on positive control samples in relation to DNA ladder bands. ***C. shasta*** **positive reactions will have an amplicon of 640 bp. Any samples not yielding this band with no apparent assay problems is reported as negative and the samples are discarded.**

    iv. If any unusual band are present in the samples or if bands are present in negative controls, re-run PCR reaction from template DNA tube.

    v. **Photo document all gels** (6.3.G) and attach the photo to the case history information (Worksheet 5.A1.C).

5.4 *Tetracapsula bryosalmonae* (Proliferative Kidney Disease)

The myxozoan (PKX), known to cause proliferative kidney disease in salmonids, is difficult to detect because its life cycle is complex, and requires a bryozoan to complete its development (Canning et al. 1999). Diagnosis of infections in fish is also complicated because the parasite does not develop completely in the fish, and resulting myxospores, if present, lack fully developed spore valves (Kent 1994). Two developmental stages occur in salmonid hosts: presporogonic stages found in blood and interstitial kidney tissue and sporogonic stages found within the lumen of kidney tubules. Although PKX-like cells have been identified from species other than in the family Salmonidae, their identity as *T. bryosalmonae* is unconfirmed.

A. Screening Tests

1. Leishman-Giemsa staining (Klontz and Chacko, 1983)
    a Stain slides with tissue imprints using Leishman-Giemsa stain.
        i The fixed imprint is incubated with approximately 1 ml Leishman stain (5.6.L) for 1 min

        ii Giemsa stain (2-3 ml)(5.6.M) is added to the Leishman stain, and allowed to stand for an additional 10-15 min.

        iii Rinse the slide and examine.

    b Whenever possible, a positive control slide should be examined prior to evaluating samples.

    c Examine a minimum of 100 fields of the stained imprints at 400x using bright field microscopy for the presporogonic stages of the parasite.

    d Presporogonic stages (Figure 5.5A) are large (approximately 20 μm) with a prominent cell membrane. The primary cell (outer, surrounding cell) contains prominent granules, a nucleus with a large nucleolus, and one or more secondary, or daughter cells. Macrophages are frequently adhered to the surface of the parasite.

    e **The finding of presporogonic stages (Figure 5.5A) is presumptive and warrants confirmation by histology**.

2. Lectin-based staining (Hedrick et al. 1992)
    a. Tissue imprints are stained with 50 μl biotinylated GS-1 lectin (L-3759, Sigma, St. Louis, Missouri) suspended in 0.01M phosphate buffer pH 6.8 (5.6.I, pH adjusted)

    b. To determine the appropriate concentration of lectin, dilutions of 25 to 0.5 μg ml^{-1} are tested on control tissue

    c. Slides are incubated in a moist chamber for 1 to 2 h at 25˚C.

d. Rinse 3 times in PBS (5.6.D).

e. Apply 50 µl fluorescein avidin D (A-2001, Vector Laboratories, Inc., Burlingame, California) diluted to provide 10-30 µg ml^{-1} suspensions in PBS.

f. Incubate slides in moist chamber at 25˚C for 30 min.

g. Rinse 3 times, then carefully blot to near dry and mount with a coverslip using a drop of a mixture containing 1 part of 0.1M N-2-hydroxy-ethylpiperzine-N'-2-ethanesulfonic acid (HEPES) pH 8.0 and 9 parts glycerol.

h. Observe under a microscope with a UV light source equipped for fluorescein.

i. **The identification of fluorescing presporogonic stages (Figure 5.5B) is presumptive and warrants corroboration by histology.**

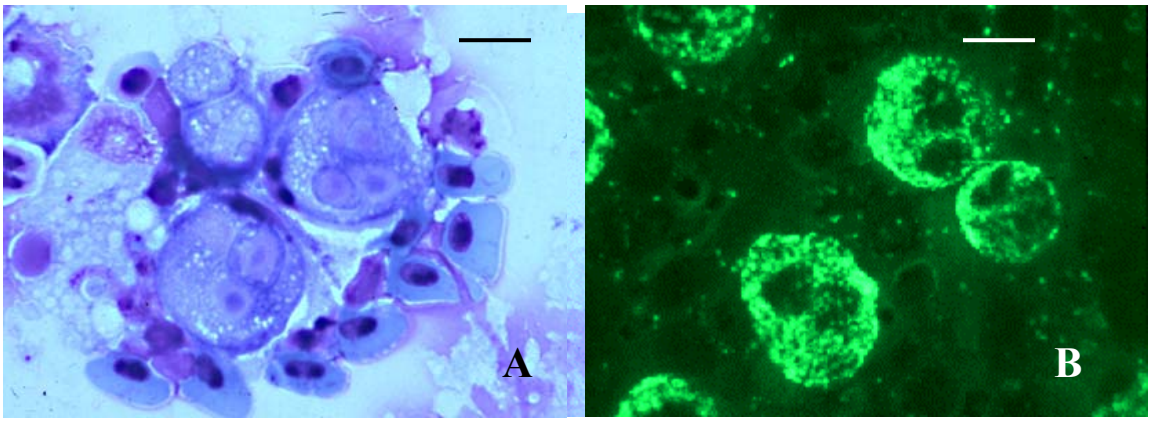


Figure 5.5. Multicellular *Tetracapsula bryosalmonae* in: A) a Leishman-Giemsa stained imprint and B) a lectin-stained imprint. Bar is 10 µm. Photographs courtesy of R. P. Hedrick.

B. Confirmatory Test

Histopathology (Hedrick et al. 1986)

1. Slides should be prepared from the kidney tissue that was placed in fixative at the time of collection using standard histological techniques.

2. Sections should be stained with hematoxylin and eosin (H&E) or Giemsa and examined for stages of *T. bryosalmonae*.

3. **The presence of presporogonic stages of *T. bryosalmonae* within the interstitial tissue of the kidney is corroboration of the infection (Figure 5.6).** A chronic inflammatory response is typically associated with the presence of the parasite (Figure 5.6B) and a "whorled" appearance may be visualized corresponding to locations of the

parasite. The presporogonic stage is approximately 20 µm in diameter and the primary cell has a lightly staining cytoplasm with a large, eosinophilic staining nucleolus (Figure 5.6C). The primary cell may contain one to several spherical, dense, secondary (daughter) cells and macrophages are frequently seen adhered to the parasite. The inflammatory cell infiltrate is primarily composed of macrophages but numerous lymphocytes are also typically present. Sporogonic stages of *T. bryosalmonae* may be observed in the lumina of kidney tubules. Morphology of spores is poorly defined in fish and it appears that hardened valves typical of other myxosporidians do not form. Valveless myxospores are approximately 12 um X 7 um and have two spherical polar capsules 2 um in diameter (Figure 5.6D).

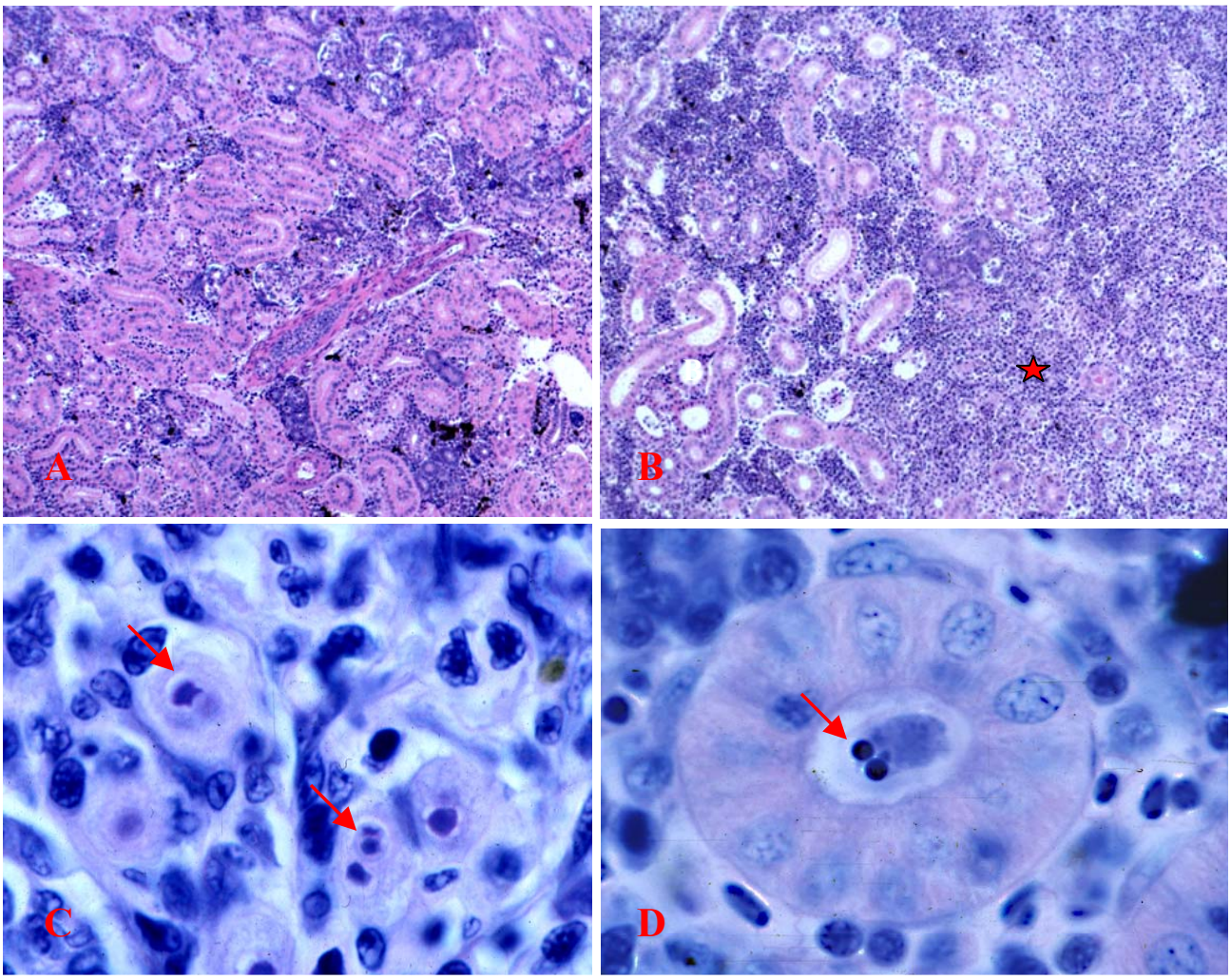


Figure 5.6. Histological sections of kidney tissue infected with *T. bryosalmonae*. a) low magnification of normal kidney, b) low magnification showing area of inflammatory cell infiltrate (★), c) high (400X) magnification of parasites in tissue and d) sporogonic stage in kidney tubule (1000X). Photographs courtesy of R. P. Hedrick.

5.5 *Bothriocephalus acheilognathi* (Bothriocephalosis, Asian tapeworm)

Bothriocephalosis is an intestinal infection of certain fish by the cestode *Bothriocephalus acheilognathi* (Mitchell 1994; Scholtz 1997), a Pseudophyllidian tapeworm. The infecting organism is also known as the Asian or Asian fish tapeworm and as the Chinese tapeworm. The Asian tapeworm has been reported in Asia, Europe, Australia, South Africa, and North America. In North America, it has been reported in Mexico, British Columbia, throughout the southern half of the United States, and in New Hampshire, New York and Hawaii. Fish become infected after ingesting infected copepods and development of the worm occurs in the anterior intestinal tract. *Bothriocephalus acheilognathi* is a thermophile that has an optimum temperature for growth and maturation above 25 ºC.

Most members of the Family Cyprinidae are considered potential hosts, with the exception of goldfish, *Carassius auratus*. Infections have also been reported in species from the following families: Siluridae, Poeciliidae, Percidae, Centrarchidae, Gobiidae and Cyprinodontidae

A. Screening Test

1. ***For fishes less than 20 cm in length*** - the uncoiled intestines from several fish may be placed side by side on a microscope slide or glass plate (9 x 9 x 0.3 cm). When intestines are too small to uncoil they can be placed as they come onto a slide. There is no need to slit the intestines. A second slide or glass plate placed over the excised intestine spreads the intestine for easy visibility. For small intestines the weight of the glass plates is usually sufficient to flatten the contents for easy viewing. Binder clips can be used if further flattening is desired (Mitchell, 1989, 1994).
    a Examination requires the use of a 15 to 30-power dissecting microscope. Reflected light gives the best results.

    b *B. acheilognathi* sometimes takes on a silvery cast and movement will be detected if the specimen is viewed for 15 seconds.

    c Thoroughly examine the anterior intestinal tract. Small worms may measure only 350 μm in length.

2. ***For fish longer than 20 cm*** - slit the anterior third of the gut open lengthwise and carefully remove any cestodes while keeping the scolex intact. Scraping the inner wall of the intestine with a scalpel ensures the collection of the scolices from visible worms and from small worms not seen. Place contents on a glass plate (see above) or microscope slide (depending on volume of contents) and cover with another plate or slide. Using binder clips, press the two plates together. Examine entire sample for *B. acheilognathi*.

3. **Visualization of a cestode found in the anterior third of the intestine that forms a pyramidal scolex in the semi contracted state (Figure 5.7) is a presumptive positive classification**. In the semi contracted state the scolex is usually 3 times the width of the segmented portion adjacent to the scolex. If large worms or many small worms are present, they may be apparent as a yellow to white bulge in the intestine.

B. Confirmatory Test

Other cestodes have similar pyramid shaped scolices. Therefore, a key must be used to definitively identify *B. acheilognathi*. A complete key for the identification of *B. acheilognathi* and accompanying figures are found in the 4th Edition of the Bluebook (Thoesen 1994). Specific characteristics needed for definitive identification are noted below.

1. ***B. acheilognathi*** - is a complete and distinctly segmented, thin tapeworm that can reach a length of over 50 cm, but is usually less than 10 cm. Segmentation is evident on worms 1 mm or more in length (Figure 5.7).

2. ***B. acheilognathi*** - has a flattened scolex with two bothria (deep, elongated sucking grooves dorsal and ventral as seen in Figure 5.7, no hooks, no spines, no true suckers, and no proboscides (short tentacles). In the lateral view, the scolex takes a strong arrowhead appearance when semi contracted and a balled or squared appearance when fully contracted. The posterior portion of the scolex is wider than the first few segments in both the extended and contracted positions.

3. ***B. acheilognathi*** - has no neck and no dorsal or ventral median furrow.

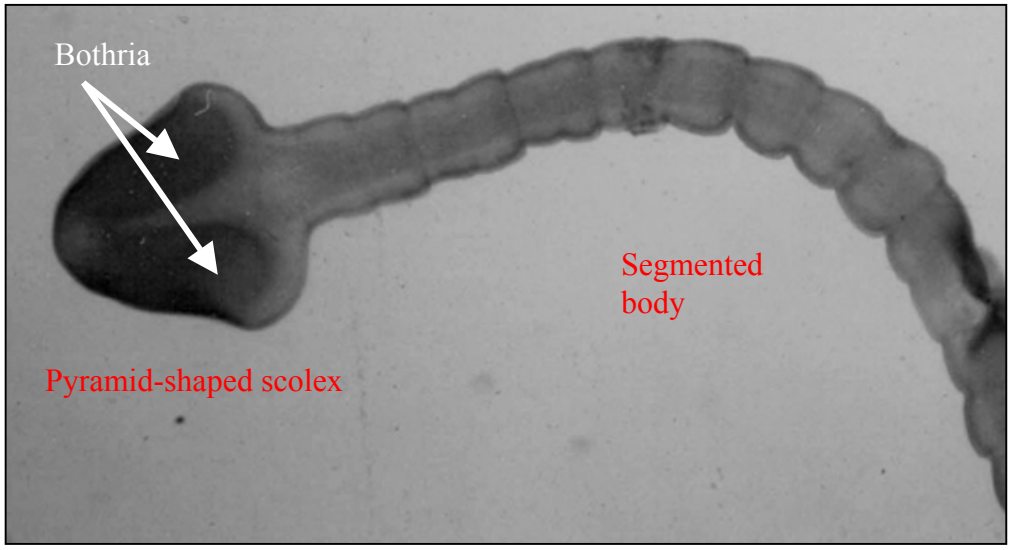


Figure 5.7: *Bothriocephalus acheilognathi* showing a pyramid shaped scolex, segmentation, and two bothria. Photograph courtesy of A. Mitchell.

5.6 Media and Reagents

A. 0.5% pepsin solution

Pepsin	5.0 g
HCl	5.0 ml
Sterile Distilled Water	to 1000 ml

Store at 4°C.

B. 0.5% Trypsin solution

EDTA	0.2 g
NaCl	8.0 g
KCl	0.2 g
KH2PO4	0.2 g
NaHPO4	1.15 g
Trypsin	5.0 g
Sterile Distilled Water	to 1000 ml

Store at 4°C.

C. 1N Sodium Hydroxide (NaOH)

NaOH	40.0 g
Distilled Water	to 1000 ml

D. Phosphate Buffered Saline (PBS)

Sodium Chloride (NaCl)	8.0 gm
Monopotassium phosphate (KH_2PO_4)	0.34 gm
Dipotassium phosphate (K_2HPO_4)	1.22 gm
Distilled water	to 1000 ml

Filter with 0.22 um filter. Store at room temperature.

E. Proteinase K

Can be obtained as a stable liquid solution (14-22 $mg.ml^{-1}$) from commercial sources. If kept at 4C, it is stable for >1 year.

F. RNAse A

It is a 10 $mg.ml^{-1}$ solution and can be obtained from commercial sources [e.g. 5'-3' (Cat. # is 5-888777)] as a 50% glycerol solution that is liquid at -20C.

G. DNA Extraction Buffer

The buffer is NaCl 100 mM, Tris-HCl 10 mM, EDTA 25 mM, SDS 1%. The stock solutions are:

NaCl 5M	{50X}
Tris-HCl 1M, pH 7.8	{100X}
EDTA 0.5M, pH 8	{20X}
SDS 20%	{20X}

Stock solutions should be made using ultrapure, nuclease-free water (HPLC grade or

equivalent), aliquoted and stored at -20 ^{o}C until needed. Pre-made stock solutions (molecular biology grade) can be purchased from a commercial supplier, aliquoted and frozen, so the chances of contamination are reduced.

H. Loading Buffer

Sigma P-7206 Pre-made 6X concentrate, ready to use (store -20C)

OR

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30.0%

I. 0.01 M phosphate buffer pH 6.8

adjust pH of PBS recipe 5.6.D to 6.8

J. 10% Neutral Buffered Formalin

Formalin, concentrated	100 ml
Distilled water	900 ml
Sodium phosphate (monobasic)	4.0 g
Sodium phosphate (dibasic)	6.5 g

K. 2.5 mM Magnesium Chloride ($MgCl_2$)

It can be obtained from commercial sources and often is supplied with Taq enzyme. Purchase as molecular biology grade.

L. Leishman Stain

Purchase from commercial source.

M. Giemsa Stain

Stock solution:

Giemsa powder	1 g
Glycerin	66 ml

Mix and place in oven at 56-66˚C for 0.5 – 1 h. Add 66 ml absolute methanol after solution has cooled

Phosphate buffer, pH 6.0

Sodium phosphate (monobasic)	35 g
Sodium phosphate (dibasic)	4.84 g
Distilled water	to 4 l

Working Giemsa

Stock Giemsa	14 ml
Phosphate buffer	200 ml

Must be made fresh

5.7 Glossary

A. Myxozoan life stages

Trophozoite – vegetative, uninucleate stage with a single diploid nucleus

Presporogonic stages – multicellular parasite stages that will eventually give rise to spores. For purposes here, this encompasses stages referred to as plasmodia, sporoblasts and sporocysts.

Spore – myxospore is the mature spore that develops in the fish. It contains polar capsules, polar filaments and sporoplasm cell(s).

Sporogenesis – the process of spore formation.

B. Cestode morphology

Scolex – portion of a cestode that bears the organs of attachment

Bothria – organ of attachment; characteristically they are long narrow grooves of weak muscularity; may become flattened to form an efficient sucking organ.

5.8 References

Andree, K. B., MacConnell, E., Hedrick, R. P. (1998). A polymerase chain reaction test for detection of *Myxobolus cerebralis,* the causative agent of salmonid whirling disease in fish, and a comparison to existing detection techniques. Diseases of Aquatic Organisms 34(2): 145-154.

Bartholomew, J. L., M. J. Whipple, D. G. Stevens and J. L. Fryer. 1997. Role of the freshwater polychaete, *Mayanukia speciosa*, in the life cycle of *Ceratomyxa shasta*, a myxosporean parasite of salmon and trout. American Journal of Parasitology. 83:859-868.

Bartholomew, J.L., J.S. Rohovec, and J.L. Fryer. 1989a. *Ceratomyxa shasta,* a myxosporean parasite of salmonids. U.S. Fish and Wildlife Service. Fish Disease Leaflet 80: 8 pp.

Bartholomew, J. L. 2001. Salmonid ceratomyxosis. *in*: ed. J Thoesen, Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. Blue Book 4th Edition. Fish Health Section, American Fisheries Society.

Canning, E. U., A. Curry, S. W. Feist, M. Longshaw and B. Okamura. 1999. *Tetracapsula bryosalmonae*. N. sp. For PKX organism, the cause of PKD in salmonid fish. Bulletin of the European Association of Fish Pathologists. 19:203-206.

El-Matbouli, M., T. Fischer-Scherl and R.W. Hoffman. 1992. Present knowledge of the life cycle, taxonomy, pathology, and therapy of some myxosporea spp. important for freshwater fish. Annual Review of Fish Diseases. 3: 367-402.

Hauck, K. and S. Landin. 1997. Detection of the Whirling Disease agent (Myxobolus cerebralis). In T.R. Meyers, editor. Fish pathology section laboratory manual. Special Publication No. 12. Alaska Department of Fish and Game, Juneau, Alaska.

Hedrick, R. P., M. L. Kent and C. E. Smith. 1986. Proliferative kidney disease in salmonid fishes. Fish Disease Leaflet 74. US Fish and Wildlife Service, Washington, D.C.

Hedrick, R. P., M. Marin, M. Castagnaro, D. Monge and P. de Kinkelin. 1992. Rapid lectin-based staining procedure for the detection of the myxosporean causing proliferative kidney disease in salmonid fish. Diseases of Aquatic Organisms 13:129-132.

Hedrick, R. P. T. S. McDowell, M. Gay, G. D. Marty, M. P. Georgiadis, and E. MacConnell. 1999a. Comparative susceptibility of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) to *Myxobolus cerebralis* the cause of salmonid whirling disease. Diseases of Aquatic Organisms 37:173-183

Hedrick, R. P., T. S. McDowell, K. Mukkatira, M. P. Georgiadis, and E. MacConnell. 1999b. Susceptibility of selected inland salmonids to experimentally induced infections with *Myxobolus cerebralis*, the causative agent of whirling disease. Journal of Aquatic Animal Health 11:330-339.

Hedrick, R. P., T. S. McDowell, K. Mukkatira, M. P. Georgiadis, and E. MacConnell. 2001. Susceptibility of three species of anadromous salmonids to experimentally induced infections with *Myxobolus cerebralis,* the causative agent of whirling disease. Journal of Aquatic Animal Health 13:43-50.

Kent, M. 1994. Proliferative Kidney Disease. *in*: ed. J Thoesen, Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. Blue Book 4th Edition. Fish Health Section, American Fisheries Society.

Klontz, G. W. and A. J. Chacko, 1983. Methods to detect the organism causing proliferative kidney disease in salmonids. Bulletin of the European Association of Fish Pathologists 3:33-36.

Lorz, H. V. and A. Amandi. 1994. Whirling Disease of Salmonids *in*: ed. J Thoesen, Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. Blue Book 4th Edition. Fish Health Section, American Fisheries Society.

Luna, L.G. editor. 1968. Manual of histologic staining methods of the Armed Forces Institute of Pathology. 3rd Edition. McGraw-Hill Book Company, New York.

MacConnell, E. and E. R. Vincent. 2002. The effects of *Myxobolus cerebralis* on the salmonid host. Pages 95-108 *in:* J. L. Bartholomew and J. C. Wilson, editors. Whirling Disease Reviews and Current Topics. America Fisheries Society, Symposium Number 29, Bethesda, Maryland

Markiw, M. E. and K. Wolf. 1974. *Myxosoma cerebralis*: Comparitive sensitivity of spore detection methods. Journal of the Fisheries Research Board of Canada. 31: 1597-1600.

Mitchell, A. J. 1989. Squash plate technique for detecting Asian tapeworms in fish. Journal of Aquatic Animal Health 1:243-244.

Mitchell, A. 1994. *in*: ed. J Thoesen, Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. Blue Book 4th Edition. Fish Health Section, American Fisheries Society

O'Grodnick, J. J., 1979. Susceptibility of various salmonids to whirling disease (*Myxosoma cerebralis*). Transactions of the American Fisheries Society 108: 187-190.

Palenzuela, O., G. Trobridge and J. L. Bartholomew. 1999. Development of a polymerase chain reaction diagnostic assay for *Ceratomyxa shasta*, a myxosporean parasite of salmonid fish. Diseases of Aquatic Organisms. 36:45-51.

Palenzuela, O. and J. L. Bartholomew. 2002. Molecular Tools for the Diagnosis of *Ceratomyxa shasta* (Myxozoa). *in:* ed. C. Cunningham, Molecular Diagnosis of Salmonid Diseases. Kluwer Academic Publishers.

Schisler, G. J., E. P. Bergersen, P. G. Walker, J. Wood, and J. Epp. 2001. Comparison of single-round polymerase chain reaction (PCR) and pepsin-trypsin digest (PTD) methods for detection of *Myxobolus cerebralis*. Diseases of Aquatic Organisms. 45: 109-114.

Scholz, T. 1997. A revision of the species of Bothriocephalus Rudolphi1808 (Cestoda: Pseudophyllidea) parasitic in American freshwater fishes. Systematic Parasitology 36: 85-107

Thoesen, J. 1994. Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens. Blue Book 4th Edition. Fish Health Section, American Fisheries Society

Wolf, K., and M. E. Markiw. 1984. Biology contravenes taxonomy in the Myxozoa: New discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449-1452.

5.A1 PCR Worksheets

A. Worksheet A – PCR Sample Data/Log Sheet

PCR Sample Data/Log Sheet

Case Number_____ **Sample Site**_____ **Date**_____
Species_____
Tissue type_____

Tissue Sample ID	Extraction Method	PCR Result	Notes
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			
12.			
13.			
14.			
15.			
16.			
17.			
18.			
19.			
20.			

B. Worksheet B - Amplification of Nucleic Acid by PCR for the Confirmation of Parasitic Fish Pathogens

1. *Myxobolus cerebralis*

WORKSHEET 5.A1.B.1: *Myxobolus cerebralis*.

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 50µl)	Volume for ____samples
d-H_2O*		Add to total 48µL		33.1	
10XBuffer		1X	10X	5.0	
$MgCL_2$		1.5mM	50mM	5.0	
dNTP's		0.4mM	5mM	4	
(+)Primer		0.8µM	20µM	2	
(-)Primer		0.8µM	20µM	2	
TAQ		2 units/Rx	5U/µl	0.4	
DNA		-	-	2 µl	-

*Add water to Master Mix first, TAQ last.

Primer Sets

Tr5-16 (round 1 frwd)	5'-GCA TTG GTT TAC GCT GAT GTA GC-3'
Tr3-16 (round 1 revs)	5'-GAA TCG CCG AAA CAA TCA TCG AGC TA-3'
Tr5-17 (round 2 frwd)	5'-GCC CTA TTA ACT AGT TGG TAG TAT AGA AGC-3'
Tr3-17 (round 2 revs)	5'-GGC ACA CTA CTC CAA CAC TGA ATT TG-3'

Control Information

POSITIVE CONTROLS		**NEGATIVE CONTROLS**	
Extraction control	PCR control	Extraction control	PCR control

Amplification

Round Number (Date & time)	**Program #**	**NOTES**

Gel Preparation

Gel Concentration		Weight of agarose (grams)	Volume of Buffer (ml)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

2. *Ceratomyxa shasta*

WORKSHEET 5.A1.B.2: *Ceratomyxa shasta*.

Case Number_____ **Date**_____

PCR Reagent	Lot#	Final Concentration	Stock Concentration	Volume per Reaction (µL) (to total 20µL)	Volume for ____samples
d-H_2O*		Add to total 19µL		14.6	
10XBuffer		1X	10X	2.0	
$MgCL_2$		2.0mM	25mM	1.6	
dNTP's		0.2mM each	10mM each	0.4	
(+)Primer		0.5µM	50µM	0.1	
(-)Primer		0.5µM	50µM	0.1	
TAQ		1 U/Rx	5U/µL	0.2	
DNA		-	-	1	-

*Add water to Master Mix first, TAQ last.

Primer Sets

Forward	Reverse
5' GGG CCT TAA AAC CCA GTA G 3'	5' CCG TTT CAG GTT AGT TACT TG 3'

Control Information

POSITIVE CONTROLS		NEGATIVE CONTROLS	
Extraction control	PCR control	Extraction control	PCR control

Amplification (Thermocycle Process)

Date & time	Program #	NOTES

Gel Preparation

Gel Concentration	Apparatus and Gel Size (Mini = 8-22 wells; Midi = 22-40)	Weight of agarose (grams)	Volume of Buffer (ml)

Gel Template (Sample Placement Map)

Ladder Brand / Lot # _____ Loading Buffer Brand / Lot # _____

Enter Sample ID below corresponding well number:

PCR Products (Loaded LEFT to RIGHT)														
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
16	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**

C. Worksheet C - Photodocumentation of the PCR Product Gel

Case Number _____ Date:

Samples

Affix gel photo documentation to this sheet and make notes on results:

AFFIX PHOTODOCUMENTATION

Notes:

Chapter 6

Polymerase Chain Reaction (PCR)

6.1 Introduction

The Polymerase Chain Reaction technique employs oligonucleotide primers to amplify segments of genes specific for the target pathogen. Reverse Transcriptase-PCR (RT-PCR) employs an initial reverse transcription step so that complimentary DNA can be amplified from viral RNA. DNA or RNA is extracted from various fish tissues and laboratory assay products, such as cell culture supernatant containing viral agents, and amplified using forward and reverse primer sets. In some instances for either method, the initial amplified product may be re-amplified using an additional "nested PCR" technique. The DNA products are then visualized by gel electrophoresis.

Specific details for sample preparation, DNA/RNA extraction, primers and cycle conditions appear in each chapter under the specific pathogens. However, certain PCR protocols and precautions are pertinent to all assays and these are described in this chapter. The PCR quality assurance and control procedures outlined below are extremely important when performing all PCR assays. (Ennis et al. 1990)

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U.S. Fish and Wildlife Service, the United States government, and/or the American Fisheries Society. Any comparable instrument, laboratory supply or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

6.2 PCR – Quality Assurance/Quality Control

A. General Considerations:

1. Quality control is critical to all steps of the PCR process, beginning with collection of samples in the field. It is important that the person performing sample collection use the precautions outlined in Chapter 2 to avoid cross-contamination.

2. Work surfaces should be decontaminated by washing with 10% chlorine (or commercial reagents like "DNA Away") to hydrolyze possible DNA contaminants. All sample racks and reusable equipment should be washed in DNA-away and autoclaved after use. Spray/wipe pipettors and working areas with DNA or RNAase -Away and turn UV on for at least 30 minutes after use (UV light damages DNA).

3. **Wear and change gloves often**. This helps prevent spread of amplified DNA or contamination of sample DNA with nucleases naturally occurring on the skin that will degrade the sample DNA. Always change to a fresh pair when leaving and entering PCR reagent mixing areas. Change gloves whenever contamination between samples is possible.

4. Employ aerosol resistant pipette tips and/or positive displacement pipettors during all extraction and amplification procedures. Separate pipettors should be dedicated for use with reagents only and another set for use with amplified products only.

5. Mix and aliquot pre-amplification ingredients under Bench top UV cabinet and NEVER contaminate this area with sample material or amplified DNA product.

6. **One aerosol drop of PCR product may contain thousands of strands of DNA, which can easily contaminate reagents!** Therefore, three separate areas of lab space are necessary to reduce the risk of contamination:
    a **Master Mix (MM) area** with UV hood **-** for mixing and aliquotting master mix reagents. Supply area with dedicated pipetters, ideally positive displacement pipettor/tips. **No samples or amplified DNA is to be handled in or near this area!**

    b **Sample Loading Area** with UV hood and dedicated pipettor - for loading of extracted (template) DNA from samples**.**

    c **Amplified DNA Area** - supplied with pipetter dedicated for **amplified PCR product ONLY.** Handle any amplified PCR products in this area only, and clean area and equipment thoroughly with "DNA-away" type solutions after working with amplified DNA.

7. Provide separate storage areas for RNA and DNA samples, amplified DNA, and PCR reagents.

8. Controls:
    a **Extraction controls** – a known positive tissue sample (or tissue spiked with target pathogen DNA) and a known negative tissue should be processed with the test samples to ensure that the DNA extraction was successful and contamination did not occur.

b **PCR controls** - sterile water (negative) and the known positive DNA and negative controls from previous extraction (positive) will ensure that the PCR process was successful and that contamination did not occur.

9. Primers: Newly received primer batches should first be tested on known positive/negative controls.

10. Dispose of trash containing amplified DNA products frequently.

B. Sample processing

1. Tissue samples should be collected on a clean bench-top, which has been disinfected using a 10% chlorine (or "DNA Away") solution if possible. If collected in the field, use a disposable work surface between each lot of tissue collected (paper towel, foil etc.)

2. Use sterile collection utensils between each lot of fish tissue collected. If data from individuals is of concern, use separate utensils for each individual. **Alcohol will not effectively decontaminate DNA from utensils.** If individual utensils are not available, flaming metal utensils between samples will effectively remove contaminants from previous samples.

3. Keep samples cold and freeze as soon as possible at or below -20^{o}C until processing can be accomplished.

4. RNA is extremely sensitive to enzymes present in sample tissues. Samples collected for RT-PCR should be frozen immediately, and transported on dry ice. An RNA stabilizing buffer can also be used and does not require that samples be frozen immediately.

C. Extraction of DNA or RNA from samples – Individual protocols will vary in specific steps for extraction of genetic material; however, the following general considerations should be employed:

1. Use micro centrifuge tubes with locking or screw-cap lids. Heating of extraction solutions causes unlocked caps to pop open, releasing aerosols that can cause cross-contamination between samples and controls. Pulse spin in the microcentrifuge before opening DNA sample tubes so that the lids are dry before opening them. This will help in preventing cross-contamination.

2. Use the accurate amount of tissue suggested by the extraction kit manufacturers. If this is exceeded, proper lysis of tissues will not be accomplished.

3. Always run positive control samples as well as negative (water and negative tissue samples) from the start of the extraction process, through amplification to electrophoresis. These controls will allow for detection of contamination as well as assure that the extraction was successful. This is the only means of assuring validity of the assay and its results.

4. Quantitation of DNA – if the protocol used advises that extracted products be measured using a spectrophotometer to ensure that enough DNA or RNA was successfully extracted, refer to quantification guidelines in 6.5.

D. Interpretation of PCR Results – Use of the appropriate controls should allow you to assess the integrity of your PCR result.

1. False-negative reactions may result from insufficient DNA extraction, excessive amounts of DNA, PCR inhibition, improper optimization of the PCR or human error (e.g. loading errors).

2. False-positive reactions may result from contamination either directly from the sample lot being tested, or from previously amplified target DNA.

3. For further help in trouble shooting see: PCR Protocols: A Guide to Methods and Applications (Ennis et al. 1990).

6.3 PCR Protocols

A. Preparation of amplification reaction mixture – Specific amplification protocols may require one or two amplification reactions.

> **Note:** samples and reagents should be kept cold either on ice or in a frozen cryo-rack during all assembly procedures.

1. Using Worksheet A "DNA Samples" (3.A3, 4.A1, 5.A1), Record appropriate data for each sample to be tested by PCR.

2. Under UV Cabinet, prepare "Master Mix" (MM) using pathogen-specific protocols in Worksheets in each chapter. Calculate the amount of each reagent to go into the MM according the number of samples to be processed. Add PCR reagents, **except for sample DNA,** in the order listed on Worksheet, adding water first and Taq polymerase last. Keep all reagents cold in frozen cryo-rack or on ice during mixing, and return them to freezer immediately after use.

   > **Note:** Prepare enough MM for 3 more samples than actually being tested to compensate for retention of solution in pipette tips and tube.

3. Place specified volume of MM into each PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

4. In the sample preparation area, load specified volume of each sample DNA to the appropriately labeled PCR tubes. To avoid cross contamination, always change tips between samples and avoid touching the sides of the tube. Close caps tightly.

B. Running the PCR - All general considerations should be employed including the following:

1. Load the sample tubes into the wells (follow manufacturer's recommendations).

2. Program thermocycler for appropriate cycle conditions and run reaction.

3. Before loading into thermocycler, give tubes a "quick-spin" to ensure that all reagents and sample are drawn down from sides of tube.

4. Thermocycler should be programmed for the specific PCR condition used for each pathogen (details under pathogen).

5. After cycling, tubes may have a ring of condensation near rim of cap. Before opening tubes, perform a "quick-spin" to draw this fluid down into the reaction area of the tube and reduce the possibility of aerosol contamination upon opening tubes.

6. PCR products can be refrigerated for up to a month following amplification (or for longer storage they may be frozen at –20˚C)

C. Detection of Product - Procedures for preparing the gel (refer to specific manufacturer's guidelines for preparation of gels and electrophoresis chambers):

1. Assemble the gel tray and position well comb in the tray according to manufacturer recommendations.

2. Prepare 1 X TAE (6.4) buffer with distilled water to volume adequate for gel and running buffer.

3. Prepare 1.5 - 2% agarose gel according to the volume recommended for specific gel forms used.
    a Weigh appropriate amount of agarose and add to proper volume 1X TAE buffer.

    b Heat solution to near boiling until agarose is completely dissolved.

    c Allow solution to cool to about 65^{o}C, then pour agarose solution into gel tray. Avoid the formation of bubbles.

    d Allow gel to cool completely for about 30 minutes, and then carefully remove the comb.

    e Position the gel into the chamber with the sample end (wells) positioned closest to the negative (black) electrode.

    f Slowly fill the chamber with the remaining 1 X TAE buffer solution until the top of the gel surface is submerged.

4. Load samples into wells as indicated for each assay.
    a For each tube of PCR product to be visualized, mix 2 µl of gel loading dye (6.4) to every 10 µl of PCR product needed to fill each well formed in the gel. Mix the sample and the dye by repeated expulsion prior to loading.

    b When the sample and the dye are adequately mixed, carefully place the pipette tip containing the mixture over an individual well of the agarose gel, and load the well with the sample. Repeat this procedure for all the wells, being sure to include the DNA molecular weight standard (one with bands at 100 bp increments in the 100-1,000 bp range) for base pair (bp) reference and positive and negative controls

    c Document gel lane assignments for each sample and control on Worksheet B, and allow for at least one lane for a DNA ladder reference.

D. Electrophoresis - approximately 80 volts for 35 minutes or until tracking dye front approaches the edge of the gel (this is dependent on gel width, so refer to manufacturer's recommendations).

E. Staining the Gel – Remove gel and tray and place in ethidium bromide (EtBr) solution (6.4) for 15 to 20 minutes.

> **Note:** EtBr solution can be reused and stored in a dark plastic tray container with a secure lid. **EtBr is very toxic** and binds with all DNA (including yours); follow appropriate manufacturer warnings! For safe proper disposal of expired EthBr solutions see Sambrook et al. or check with your local biotech supply retailer for specific products designed to remove EthBr from solution for disposal.

F. De-staining the Gel - in water for 5 to 60 minutes. De-stain water should be handled and disposed of appropriately (see 6.3.E)

G. Visualize the DNA

1. Place gel on a UV light source and carefully record locations of bands on positive control samples in relation to the DNA molecular weight standard. Band locations of positive controls should be at anticipated locations according to primers used.

   Note: Use UV protective goggles or face shield

2. Note any unusual band occurrences. Negative controls should not have any bands. Contamination suspicions indicate the samples should be re-run from template DNA tube.

3. Photo document all PCR gels and attach to Worksheet B (or provide reference for finding the photo documentation).

6.4 Reagents

REAGENT:	REFERENCE:	DIRECTIONS TO PREPARE:
TAE BUFFER 10X	Sigma T-4035	Comes in prepared packets, add DI water & qs to 1L . Label as 10X- STOCK (store at RT). Also can prepare a 50X stock (see Sambrook, et al.)
TAE BUFFER 1X WORKING SLN	TAE-1X	Diluted 1:10 from 10X stock Label as TAE - 1X (store at RT)
PCR LOADING BUFFER -OR- LOADING DYE	Sigma P-7206 LOADING DYE 6X	Pre-made 6X concentrate, ready to use (store -20˚C) Prepare in-house (per Sambrook, et al -store at 4˚C): Bromophenol blue 0.25% Xylene cyanol 0.25% Glycerol 30.0 %

ETHIDIUM BROMIDE - Recommend buying EtBr already in solution to minimize working with this hazardous compound, or it can be prepared as follows:

Note: USE CAUTION IN PREPARING EtBr SOLUTIONS: Follow all MSDS precautions. Wear gloves, avoid all contact with skin, eyes, and respiratory system. LABEL ALL BOTTLES WITH "CHEMICAL CARGINOGEN".

EtBr STOCK SOLN - 10mg/ml	Ethidium Bromide 100 mg DI water 10 ml Label as EtBr STOCK (10mg/ml) Protect from light (store at RT)	
EtBr-WORKING SOLN - 4.0ug/ml* WORKING STAIN SLN	Add 200ul STOCK SLN to 500ml water Label EtBr - Working Sln (store at RT)	

*References may suggest weaker working solutions (0.5ug/ml) and staining periods of 45-60 minutes.

6.5 Analysis of Extracted DNA using an UV Spectrophotometer

DNA Quantitation is achieved routinely with the use of spectrophotometry. The 230, 260 and 280 wavelengths provide the readings for quantification and purity and the 320 wavelength provides a reading for background compensation.

Standard quantitative conversion factors for nucleic acids are as follows:

1 absorbency unit at 260 nm of ds DNA = 50 ng/ul
1 absorbency unit at 260 nm of ss DNA = 33 ng/ul
1 absorbency unit at 260 nm of ss RNA = 40 ng/ul

Absorbance values for the 260 nm readings need to be in the linear range (between 0.1 and 1) for quantitation to be valid. If the absorbance at 260 nm (A260) is above 1.0 the sample needs to be diluted. If the sample is below 0.1 there is negligible DNA in the sample.

Relative purity of the DNA sample can be determined by the A260/A280 ratio. If the sample is pure nucleic acid the ratio should be approximately 1.9. Excess protein in the sample will raise the absorbance at 280 nm thereby reducing the A260/A280 ratio.

One inexpensive instrument for this purpose is the GeneQuant II (Pharmacia Biotech) although other makes and models are available. The GeneQuant II is a spectrophotometer specific for obtaining concentrations of either double-stranded DNA (dsDNA); single-stranded DNA (ssDNA) or RNA in units of weight, molar fraction, moles of phosphate and total molecules. The instrument is capable of measuring the RNA or DNA using UV wavelengths at 230nm, 260 nm, 280 nm, and 320 nm simultaneously. If a standard spectrophotometer is all that is available then conversion to pmoles /ul (ie.- uM) from ng/ul can be accomplished with the following table.

Amount of primer (ng) needed to equal 10 pmol:

Primer Length	ng of primer equal to 10 pmol
15	50
16	53
17	56
18	59
19	63
20	66
23	78
24	80

6.6 References

Sambrook J, Fritsch EF, Maniatis T. 1987. Molecular cloning: a laboratory manual. 2nd Edition. Cold Springs Harbor Laboratory Press. Plainview, New York.

PCR Protocols: A Guide to Methods and Applications 1990. edited by M. A. Ennis, D. H. Gelfand, J. J. Sninsky, and T. J. White; Academic Press, Inc.

Appendix 1

The Handbook and Oversight Committee

A1.1 Format of The Handbook

A. Title

1. The document itself shall be titled "Standard Procedures for Aquatic Animal Health Inspections"

B. Chapters – There shall be 6 chapters plus any necessary appendices

1. Chapter 1 – Introduction - This chapter will contain introductory information and explanation about this handbook

2. Chapter 2 – Sampling - This chapter will contain information about sampling animals for a health inspection

3. Chapter 3 – Bacteriology - This chapter will contain information on sampling, testing and corroborating samples for bacterial pathogen inspection

4. Chapter 4 – Virology - This chapter will contain information on sampling, testing and corroborating samples for viral pathogen inspection

5. Chapter 5 - Parasitology - This chapter will contain information on sampling, testing and corroborating samples for parasitic pathogen inspection

6. Chapter 6 – Polymerase Chain Reaction – General Protocols – This chapter shall contain general protocols and quality assurance and quality control information for utilizing polymerase chain reaction methodologies.

7. Appendices
    a. Appendix 1 – The Handbook - This Appendix will contain the format information for the handbook and procedural information for making changes to this handbook.

    b. Appendix 2 - Handbook Committee and Sub-Committee Members

        i This appendix will keep a record of those individuals serving on any committee or sub-committee, their terms of appointment and their position on the committee.

        ii The construction of this appendix shall be as follows:
            1 Each Subchapter of this Appendix will represent one year.

            2 Each committee member will be listed as a section within the sub-chapter of the Appendix.

            3 Their position on the committee will then be listed as an item under their section

4 Subcommittee members will be listed as items under the committee member that chairs the subcommittee on which they serve

5 Entries will include the name, title, and affilitiaion of the individual

c. Appendix 3 – Position Statements - This appendix will keep a record of all discussions by those individuals serving on any committee or sub-committee, their terms of appointment and their position on the committee. The construction of this appendix will be as follows:
   i Each sub chapter of this appendix will represent one chapter in the handbook
      1 The Introduction will be Appendix 3.1

      2 Sampling will be Appendix 3.2

      3 Bacteriology will be Appendix 3.3

      4 Virology will be Appendix 3.4

      5 Parasitology will be Appendix 3.5

      6 Polymerase Chain Reaction – General Protocols will be Appendix 3.6

      7 Appendix 1 changes will be documented in Appendix 3.7

   ii Each submission will be a section within the appropriate sub-chapter of the Appendix.

   iii Each position statement and the resultant changes will then be listed as items under their section

C. Layout – The layout of the document will be as follows:
The format will be in Outline form

a. The alphanumeric system will be: 1.2, **A**, 1, a, i, *1*, *a, i*

b. Appendices will be also be in outline format with an "A" being placed before the appendix number.
   i Appendix three of the entire document would be titled and referenced as (A3)

   ii Appendix two of Chapter 3 would be titled and referenced as (3.A2)

Chapter Structure

c. Each chapter will have its own references

d. Each Chapter will have its own Appendices

e. Chapters 1 (Introduction), 2 (Sampling), 6 (Polymerase Chain Reaction – General Protocols) and the Appendices are to serve as references for the other chapters

References to other sections within the handbook will be in the same alphanumeric form as the outline.

f. Example: Reference (1.2.C.3.a) will be found in chapter 1, sub chapter 2, section C, number 3, item a.

D. Stringency

1. The appropriateness of methodologies shall be determined based on several factors to make these protocols realistic to accomplish.
   a. The sensitivity of the assay

   b. The specificity of the assay

   c. The cost of the assay.

   d. Availability of reagents

   e. Availability of technology

   f. Manpower requirements.

2. All methods and protocols provided in this handbook shall be referenced and scientifically defensible.

3. The incorporation of additional pathogens into this handbook shall be contingent on the availability of appropriate screening and COROBERATIVE tests, and because such tests are commonly required by fish health regulators.

E. Maintenance of the Handbook

1. Handbook Revision and Oversight Committee
   a. Annual review as requested by open invitation (see process below)

   g. Physical possession of the electronic editable version of the Handbook in Microsoft Word 2000 Format
      i This shall be held by four individuals

1 Handbook Revision and Oversight Committee Co-chair, United States Fish and Wildlife Service Representative

2 Handbook Revision and Oversight Committee Co-chair, American Fisheries Society – Fish Health Section Representative

3 United States Fish and Wildlife Service National fish health coordinator or their duly appointed representative.

4 Chairman of the American Fisheries Society – Fish Health Section or their duly appointed representative.

h. Maintain an appendix of position statements to keep track of discussions so as not to revisit issues that have been previously decided when no new information has come available.
    i Changes to the Handbook shall be recorded as well, so the specifics of any given change can be examined in the future.

    ii Dissenting opinions shall be included in the position statements as well

F. Distribution of the Handbook

Electronic Distribution (Non-editable) – PDF Format

a. CD Rom distribution of current version

b. Web Site
    i Initial Distribution

    ii Yearly updates

Printed Handbooks of current version.

A1.2 Handbook Revision and Oversight Committee

A. Committee Construction

The handbook revision and oversight committee shall consist of 8 people

a Three (3) from the U.S. Fish and Wildlife Service

b Three (3) from the American Fisheries Society – Fish Health Section.

c United States Fish and Wildlife Service National fish health coordinator or their duly appointed representative. (Ex-officio)

d Chairman of the American Fisheries Society – Fish Health Section or their duly appointed representative. (Ex-officio)

2. The committee will be co-chaired by one individual from each organization.

3. The terms of these committee members shall be 3 consecutive years.
   a Terms shall begin 2 months prior to the individuals first annual meeting

   b Terms shall conclude 10 months after the individuals third consecutive annual meeting.

   c An individual may not serve on the committee again once their 3 year term has expired, unless a period of 2 years has elapsed.

Each committee member will rotate through the committee as follows.

d Year one – In-coming member

e Year two – Co-chair

f Year three – Outgoing member

g This rotation will allow for continuity from year to year.

New committee members shall be invited to join the committee by the new committee co-chairs sixty (60) days prior to the annual meeting.

B. Committee Function

The Handbook Revision and Oversight Committee shall meet in person at least once a year.

a The meeting shall be held in September of each year.

b All additional or subsequent meetings of this committee or its sub-committees do not need to be held in person.

c At least 2/3 of any committee or sub-committee must be present to make official decisions.

2. The Handbook Revision and Oversight Committee shall address all requests for revision of the Handbook.

3. The Handbook Revision and Oversight Committee shall keep the Handbook current and shall update all distributables (CD-ROM, web page, and print version) at least once a year, and no later than 60 days prior to the next in person committee meeting.

4. The Handbook Revision and Oversight Committee shall maintain possession of the editable copies of the Handbook

5. The Handbook Revision and Oversight Committee shall oversee the distribution of the Handbook

A1.3 Process for Amending the Handbook (Fig. 1)

A. At least sixty (60) days prior to their annual meeting, the Revision and Oversight committee shall, through the new committee co-chairs, send out a notice to all U.S.F.W.S. project leaders and shall publish a notice in the AFS-FHS newsletter with the date of the annual review of the inspection handbook. Additionally comments and concerns should be solicited at this time for discussion at the meeting. Individuals should be encouraged to include any new information that may need to be considered when evaluating their comments and concerns.

B. Written requests for changes in the document or for discussion of concerns shall be submitted no later than thirty (30) days prior to the annual meeting of the revision and oversight committee. The requests and comments shall be submitted to the committee co-chairs who will then distribute them to the other committee members for their consideration prior to the meeting.

C. Committee members shall search the appendix for related issues or position statements that have addressed these concerns or similar concerns. This shall be done to aid in determining the need to address a particular query due to changes in available information.

D. At the annual meeting the committee will hold discussions on each query to determine whether or not the issue merits further review.
   1. If the committee calls for no further review, the committee shall write a position statement to be incorporated with the query and added to Appendix 3. Additionally the person submitting the query shall also receive a copy of the position statement.

   2. Should the committee decide the matter warrants further discussion a sub-committee shall be formed.
      a. Sub-committees shall be formed with regard to the chapter under review (ie. Bacteriology, Virology, and Parasitology), this will ensure consistency within any chapter. Sub-committees formed to deal with issues within Chapter 1, Chapter 2, Chapter 6 or Appendix 1 should focus on how changes will affect all sections of the handbook as these relate to all the other chapters.

      b. A member of the Revision and Oversight Committee shall chair each sub-committee.

      c. Each sub-committee will address all the queries regarding their chapter moved forward by the Revision and Oversight Committee

      d. The sub-committees shall be composed of 6-12 members.
         i. Members are drawn from both the USFWS and the AFS-FHS.

ii. Equal representation is not required on sub-committees; however there must be at least two representatives of each organization on each sub-committee.

iii. The sub-committee chair will invite individuals to participate on the sub-committee.

iv. The invitees shall be experts or have extensive experience in the discipline being discussed.

v. The invitees shall represent a diverse area of the country and have a broad species interest, to ensure equal consideration of all potential culture situations.

e. The sub-committee chair shall assemble the sub-committee, provide them with the queries to be addressed and any other material needed and schedule a meeting within 30 days of the annual meeting.

E. The sub-committees shall determine again if the query needs to be addressed.
If it is determined by the sub-committee that the issue does not warrant action:

a The sub-committee shall write a position statement explaining why this decision was made and it will be submitted to the Revision and Oversight Committee for inclusion in Appendix 3.

b The submitter of the query will also be provided a copy of the position from the sub-committee.

If the sub-committee finds the query needs to be addressed they shall:

a. Determine the most appropriate changes to be made to the document

b. Submit line-by-line recommendations in a position statement, which is delivered to the revision and oversight committee within ninety (90) days of the sub-committees first meeting.

F. At this time the Revision and Oversight Committee shall have thirty (30) days to review these changes and determine if they fit with the other parts of the handbook and are in-line with the purpose and intent of the handbook.
If the committee accepts them:

a The revision and oversight committee co-chairs make the changes to the handbook.

b The changes as well as the sub-committee position statement are added to Appendix 3.

c The individual submitting the query shall receive a copy of the position statement and notice of the subsequent changes to the handbook.

2. If the revision and oversight committee rejects the suggested changes. They shall supply a written position statement for these actions to the subcommittee chair, who in turn shall provide it to the sub-committee members.

G. The sub-committee shall have an additional sixty (60) days to:
   1. Resubmit their changes, by submitting a new position statement to the Revision and Oversight Committee through the sub-committee chair.

   2. Drop the changes, and issue a new position statement for inclusion in Chapter 3.

H. The revision and oversight committee shall have thirty (30) days to re-review these changes.
   If the new submittal is accepted by the Revision and Oversight Committee they shall:
      a Write a position statement

      b Make the changes to the handbook

      c Add the changes well as all position statements to Appendix 3.

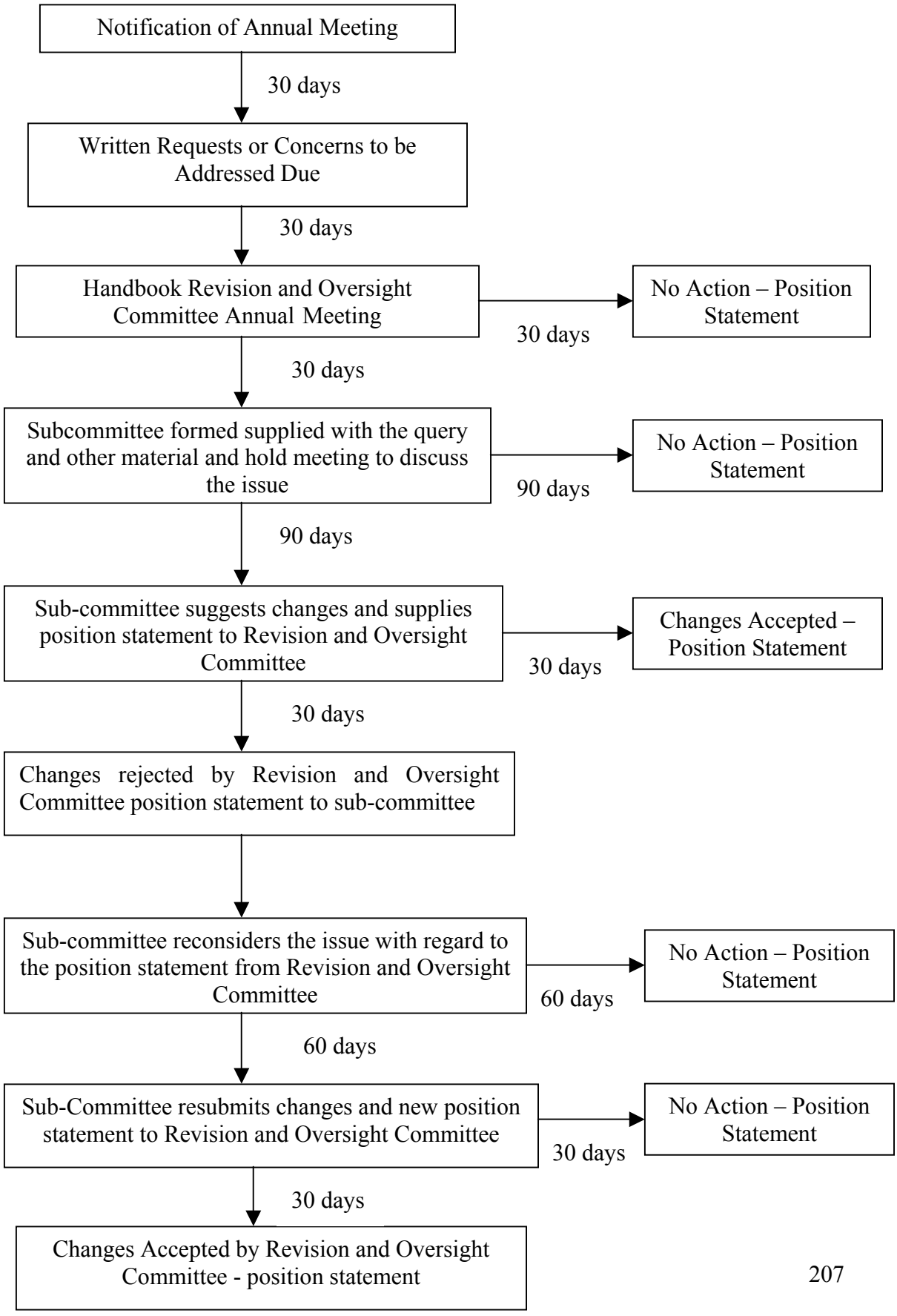
      d Supply the query submitter a copy of both the position statement and the subsequent changes to the handbook.

   If the submission is once again rejected the revision and oversight committee shall:
      e Provide a position statement, which shall be included in Appendix 3 and a copy provided to the individual that made the request.

      f The request will then be considered closed and will only be reconsidered if re-submitted in the future.

I. Position statements may be written by any member of the sub-committee or Revision and Oversight Committee and signed off on by all committee members before being added to Appendix 3. It is essential that all opinions, including those dissenting from the decision, be included in the position statement.

Figure 1: Flow Chart of procedures to handle requests to change the handbook



Appendix 2

Handbook Committee Members

A2.1 2000 – 2002 (Handbook Development Committee)

A. Dr. Robert S. Bakal – USFWS, Warm Springs Fish Health Center, Co-chair, General Editor

B. Dr. Jerri Bartholomew – Oregon State University, Co-chair, Parasitology Subcommittee Chair

1. Dr. Karl Andree - University of California-Davis, Dept. Veterinary Medicine and Epidemiology
2. Dr. Andrew Goodwin - Aquaculture/Fisheries Center, University of Arkansas at Pine Bluff
3. Ms. Kimberly True – USFWS, California – Nevada Fish Health Center
4. Ms. Linda Vannest – USFWS, Bozeman Fish Health Center
5. Dr. Becky Lasee – USFWS, La Crosse Fish Health Center
6. Dr. Linda Chittum - Utah Division of Wildlife Resources (Served as subcommittee chair until needing to step down)
7. Ms. Elizabeth MacConnell – USFWS, Bozeman Fish Health Center

C. Ms. Patricia Barbash – USFWS, Lamar Fish Health Center, Bacteriology Subcommittee Chair

1. Dr. Marilyn Blair - USFWS, Idaho Fish Health Center
2. Dr. Larry Hanson – College of Veterinary Medicine, Mississippi State University
3. Mr. Phillip Hines - USFWS, Pinetop Fish Health Center
4. Mr. John Hnath - Michigan Department of Natural Resources
5. Ms. Susan Marcquenski - Wisconsin Department of Natural Resources
6. Mr. David Money – Wyoming Game and Fish, University of Wyoming
7. Mr. Ronald Pascho - USGS, Western Fisheries Research Center
8. Mr. Eric Pelton - USFWS, Lower Columbia Fish Health Center
9. Dr. Clifford Starliper - USGS, National Fish Health Research Laboratory
10. Dr. Chris Wilson - Utah Division of Wildlife Resources

11. Dr. Doug Eib - New Mexico Department of Natural Resources

D. Dr. Joy Evered – USFWS – Olympia Fish Health Center, Virology Subcommittee Chair

1. Kevin Amos – NMFS, National Seafood Inspection Laboratory and Washington State Department of Fish and Wildlife
2. Mr. William Batts - USGS, Western Fisheries Research Center
3. Dr. Scott LaPatra – Clear Springs Foods, Inc.
4. Mr. Ken Peters – USFWS, Bozeman Fish Health Center
5. Dr. John Plumb – Department of Fisheries and Allied Aquaculture, Auburn University
6. Mr. Bruce Stewart – Northwest Indian Fisheries Commission
7. Dr. Jim Winton - USGS, Western Fisheries Research Center

Appendix 3

Position Statements

A3.1 Introduction

A. 2000 – 2002 (Initial Position Statement)

Position Statement

This chapter sums up why and how we have assembled this document. We have tried to explain the purpose and makeup of the document so there is as little confusion as possible. We had to recognize there was no way to please everyone, when so many people with differing needs and wants will use the document. We have done our best to provide what we believe is the best possible document at this time. We have adhered very closely to OIE guidelines to make this document as useful as possible for international trade inspections. We are hopeful this document will continue to grow and evolve. . Individual jurisdictions are likely to require different criteria for an aquatic animal health inspection and those criteria shall supercede the recommendations set forth in this chapter.

A3.2 Sampling

A. 2000 – 2002 (Initial Position Statement)

Position Statement

This chapter was by far the most difficult to develop, due to the vast nature of situations and scenarios an inspector might come across. It is impossible to cover all scenarios and situations; therefore while we have done our best to cover as much as we can it will remain incumbent on the inspector to determine how best to sample in any given situation. Individual jurisdictions are likely to require different criteria and those criteria shall supercede the recommendations set forth in this chapter.

A3.3 Bacteriology

A. 2000 – 2002 (Initial Position Statement)

Position Statement

The pathogens selected were those the committee felt were of the greatest regulatory importance at the time the handbook was being developed. The four bacterial fish pathogens considered in this chapter represent etiological agents which are known to exist in carrier states, but which have the potential for generating severe epizootics of clinical disease under the appropriate conditions. The methods are described for detection and identification of each pathogen in the absence of clinical signs. While other bacterial pathogens exist which can cause serious disease in fish, they are often widely distributed and cannot be controlled through transfer restrictions due to their ubiquitous nature (such as the pathogenic *Flavobacterium* species), and therefore are not the focus of these inspection procedures.

The accurate identification of a bacterial species is based upon patterns of characteristics observed when live, pure bacterial isolates are cultured under a variety of environmental and biochemical conditions. All four bacterial fish pathogens considered for identification during a Fish Health Inspection are culturable. All have been exhaustively characterized in a variety of widely recognized bacteriological manuals (Bergey's, 1984; MacFaddin's 1980 & 2000; Austin & Austin, 1987). The extensive characterization of these species has lead to the establishment of simple testing schemes for presumptive identification of bacteria isolated from fish tissues as described in these protocols. *Renibacterium salmoninarum ,* however, is relatively fastidious and difficult to culture and characterize phenotypically in the period of time desired to accomplish the completion of a Fish Health Inspection. Serological techniques are also considered to be rapid, highly specific means for achieving presumptive identification of bacteria. Because of its fastidious nature, the fluorescent antibody technique has been long developed as a presumptive screening tool for the detection of *R. salmoninarum* in fish tissues.

It is generally agreed that identification of a bacterial isolate based on phenotypic or serological characteristics alone poses the possibility that a population of fish be inaccurately labeled as diseased on a Fish Health Inspection Report. Although either method of identification is acceptable as a screen for pathogens in fish, neither technique alone is precise enough to distinguish between some similar organisms. For these reasons, it is always necessary to apply a second testing regime, referred to here as "confirmatory", to establish the accuracy of the screening test. The protocols described in this document are presented in such a manner. In past decades, studies with nucleic acids and genetic methods have furthered the accuracy in the classification and identification of bacterial species. These tools, however, were limited to research because of the difficulty in applying them accurately under clinical situations. The more recent developments in polymerase chain technology, however, have revolutionized the use of molecular biology in pathogen detection in clinical laboratories. PCR is a practical, sensitive and accurate means to confirm the presumptive identification of a bacterial pathogen by the isolation and amplification of

segments of DNA existing within fish tissues. It is presented in these protocols as an alternative to time consuming selective culture for confirmation of positive *R. salmoninarum* FAT results.

A3.4 Virology

A. 2000 – 2002 (Initial Position Statement)

Position Statement

The eight viral pathogens considered in this chapter represent agents that may exist in a carrier state, have the potential for causing severe epizootics and/or are currently of regulatory concern. This list will likely change as these concerns vary and new control measures are developed. Techniques provided for screening and confirmation are considered to be sensitive, practical, and efficient, and applicable to the large numbers of samples necessary to detect viral pathogens in carrier states. The potential variety of techniques is limited to cell culture for screening and serum neutralization and/or PCR for confirmation to simplify the writing of this initial Handbook. Other serological methods such as immunoblot and fluorescent antibody tests are available for some of these viruses and applications may be made to add these to later versions.

Cell culture is the screening method used and broad spectrum cell lines have been chosen whenever possible to aid the testing laboratory in getting the most information from the samples.

Blind passage of samples has been included to determine if it will significantly increase the ability of the laboratories to detect carrier stages of these viruses using these methods.

Since cell culture amplifies the virus, it allows for the use of a highly sensitive but not necessarily specific confirmation method (see Chapter 1). The utility of serum neutralization tests for the confirmation of IHNV, IPNV, SVCV, and VHSV has been shown with years of use and for that purpose it is included here, however, the reagents are not available for all of the viruses in this Handbook. PCR is a newer technique that is also highly specific but much more rapid than serum neutralization and the detailed methods for using it to confirm IHNV, ISAV, LMBV, and VHSV are also included. PCR techniques are being developed for IPNV, OMV and WSHV and applications may be made to include them in future version as the methods and reagents become available.

A3.5 Parasitology

A. 2000 – 2002 (Initial Position Statement)

1. Position Statement

The pathogens selected were those the committee felt were of the greatest regulatory importance at the time the handbook was being developed. Rationale for selection of the screening and confirmatory assays for each of the fish parasites considered in Chapter 5 are detailed below. Confirmatory procedures will only be used if the sample is presumptively positive using the approved screening method. Please refer to chapter 1 for explanation of the acceptance of non-validated procedures for confirmation.

Myxobolus cerebralis

Screening - The pepsin-trypsin digest procedure was selected as the assay of choice for isolation and concentration of spore stages from fish cartilage. Although it was acknowledged that the plankton centrifuge method offers some advantages in the ease of assay performance, review of the literature and of laboratories performing *M. cerebralis* diagnostics supported selection of the digest assay for reasons of increased sensitivity. The procedure does allow pooling of up to 5 fish, which is likely to decrease detection sensitivity. However, it was considered that processing of individual fish would constitute a workload beyond the capability of many laboratories, and that in some regions of the country this would be considered unacceptable. The decision was to allow pooling with the realization that in areas most affected by the parasite there would be requirement by the states to process single fish.

Confirmation – Confirmation is either by identification of spores in histological sections or detection of parasite DNA by polymerase chain reaction (PCR) assay. Detection in histological sections is the current standard. Although the committee felt that it is of lower sensitivity than the PCR assay, it will remain an acceptable confirmatory tool at this time. For DNA detection, the nested PCR assay was selected because it scientifically acceptable and citable and it is used successfully in a number of laboratories. Because the sampling and preparation procedures described in the original publication were primarily for research purposes, the protocol described here references methods more in line with those required during field collections of fishes of different sizes. These collection and preparation methods are compatible with performing the nested assay.

Ceratomyxa shasta

Screening – Presumptive identification is based on identification of any parasite stages in wet mount scrapings, the procedure currently recommended.

Confirmation – Because of the distinctive morphology of the *C. shasta* spore, its identification is sufficient for confirmation. If spores are not identified, a

presumptive positive can be confirmed by detection of parasite DNA by PCR. The protocol described is published and has been developed for diagnosis in field situations. Other confirmatory procedures requiring monoclonal antibodies were not considered because these reagents are not commercially available.

Tetracapsula bryosalmona

Screening – Presumptive identification is made by identifying any parasite in stained imprints or using lectins. These two methods were proposed because identification of the parasite is difficult without practice, and the lectin has been shown to increase detection.

Confirmation – At this time, confirmation is by identification of any parasite stages in histological sections. Although this method is not highly sensitive and requires a trained eye, it was agreed that scientific review of other methods made them unfeasible at this time. The lectin stain has been demonstrated to cross-react with other myxozoans and there is also question about the specificity of published PCR assays. The committee felt that this protocol would probably be updated in the near future as a demonstrated specific PCR assay becomes available.

Bothriocephalus acheilognathi

Screening – Presumptive identification is by identification of basic characteristics of the cestode.

Confirmation – Presumptive cestodes are confirmed by identification of key morphological characteristics. These visual identification methods are accepted in the scientific literature and are the current Bluebook standard.

A3.6 Polymerase Chain Reaction – General Protocols

A. 2000 – 2002 (Initial Position Statement)

Position Statement

This chapter was designed to supplement references to molecular techniques referenced in earlier chapters. Included are general considerations for insuring that contamination does not occur and to insure the integrity of the assay. These general protocols that can be found in many general primers for PCR and are intended to provide background information for laboratories that are just setting up PCR diagnostics.

A3.7 Appendix 1

A. 2000 – 2002 (Initial Position Statement)

Position Statement

This Appendix is truly the heart of this document. It lays out the structure of the handbook and the revision and oversight committee. It explains how the handbook will be maintained and by who. Most importantly, however, this Appendix details the manner in which this handbook shall be reviewed and revised. This detailed procedure is what gives this document its advantage over previous documents of its kind. Additionally, these reviews are mandatory on an annual basis, which means the document can be kept current from a technique and pathogen standpoint, such that in the future there should be no need to create any new handbooks or manuals.